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THE EFFECT OF ADDITIVES ON ENZYME PARTICLE SIZE AND DISTRIBUTION IN SPRAY DRYING

Master's thesis for the degree of Master of Science in Technology submitted for inspection, Espoo, 1st of August, 2016.

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Abstract of master's thesis

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Abstract

Enzymes and other products are dried to increase the storage life as liquid products are more vulnerable to contamination. Spray drying can be used to produce enzyme powders but they often contain fine particles. Enzymes are allergenic and thus the smallest enzyme particles can cause permanent health issues for employees exposed to them.

The aim of this study was to find ways to affect enzyme particle size by using additives as well as to reduce or remove the smallest particles from the product.

The effect of sodium chloride on enzyme particle size was studied with three enzymes. In addition, depending on the enzyme the use of sodium sulphate, calcium acetate, calcium chloride, methyl cellulose, ascorbic acid and pH was studied.

The enzyme samples were spray dried with BÜCHI Mini Spray Dryer B-290. The particle size distribution and enzyme activity were measured for all the samples, and electron microscope pictures were taken of chosen samples to receive more information.

There were significant differences between the particle size distributions of different enzymes. The largest particle sizes were received with an enzyme that precipitated easily because of additives or adjustment of pH. That enzyme also showed the greatest differences between the additives. Largest particles were around 1000 µm in diameter formed of particles with a diameter of around 10 µm. These kinds of particles are called agglomerates.

Agglomeration was discovered to be an important factor in the increase of the particle size and to reduce the number of small particles. Even though it was possible to increase the mean particle size by increasing the diameter of the particles, the differences were still significantly smaller compared to those samples that had agglomeration.

A connection between enzyme precipitation and increased agglomeration was discovered but as there were many factors, of which several unknown, that affect the final product, the analysis of the results was complicated. In order to confirm the connection, repeating the experiments is necessary.

Keywords Spray drying, enzyme, additive, particle size distribution, agglomeration, precipitation

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Tiivistelmä

Entsyymejä ja muita tuotteita kuivataan säilyvyyden lisäämiseksi, sillä nestemäisinä entsyymiliuokset ovat alttiimpia kontaminaatioille. Spray-kuivauksen tuloksena saadaan entsyymijauhetta, joka usein sisältää heinojakoisia partikkeileita. Entsyymit ovat allergeenisia, ja siksi pienimmät entsyymipartikkelit voivat aiheuttaa altistumisriskinsä vuoksi pysyviä terveyshaittoja työntekijöille.

Tässä työssä tarkoituksena oli etsiä keinoja vaikuttaa entsyymien partikkelikokoon lisäaineiden avulla. Lisäksi pienimpien partikkelien poistaminen tai vähenteminen entsyymijauheista oli merkittävä käytännön tavoite.

Työssä tutkittiin natriumkloridin vaikutusta kolmen eri entsyymin partikkelikokoon. Lisäksi entsyymistä riippuen tutkittiin natriumsulfaatin, kalsiumasetaatin, kalsiumkloridin, metyyliselluloosan ja askorbiinihapon sekä pH:n vaikutuksia.

Entsyyminäytteet lisäaineineen kuivattiin BÜCHI Mini Spray Dryer B-290 –pöytäkuivurilla. Partikkelikokojakauma ja entsyymiaktiviisuus mitattiin kaikille jauhenäytteille ja lisäksi osa näytteistä analysoitiin elektronimikroskooppikuvien avulla.

Entsyymien välillä huomattiin suuria eroja partikkelikokojakaumissa. Suurimmat partikkelikoot saatin entsyymillä, joka sakkautui lisäaineiden tai pH-olosuhteiden vaikutuksesta voimakkaimmin. Lisäaineiden välillä näkyi myös suurimmat erot kyseisellä entsyymillä. Suurimmat partikkelit olivat kokoluokkaa 1000 µm halkaisijaltaan. Ne olivat agglomeraatteja, eli ns. partikkeliryppäitä, jotka muodostuivat noin 10 µm halkaisijan partikkeleista.

Agglomeraatio todettiin merkittäväksi tekijäksi pienten partikkelien vähentämisen kannalta. Vaikka keskimääräinen partikkelikoko saatiin kasvamaan ilman merkittävää agglomeraatiota, erot olivat suhteessa merkittävästi pienempiä kuin silloin kun parkikkelikoko kasvoi agglomeraation vaikutuksesta.

Enstyymien sakkautumisen ja lisääntyneen agglomeraation välillä huomattiin yhteys, mutta monet tekijät vaikuttivat lopputuotteeseen vaikeuttaen tulosten analysointia. Toistojen vähyyden takia yhteyttä ei myöskään voitu todistaa.

Avainsanat Spray-kuivaus, entsyymi, lisäaine, partikkelikokojakauma, agglomeraatio, saostuminen

Preface

The study was performed in Roal Oy in 2016. My advisor from the company was their technical manager Arni Kujala and my supervisor from Aalto University was professor Alexander Frey from the Department of Chemical Technology. The experimental part was mostly performed in Formulation group in Roal lead by Katja Palmunen.

The motivation for this study was to increase safety with enzyme powders by reducing the number of small particles using additives in spray drying.

I want to thank you everyone who took part in my project and especially Arni Kujala for advising, Katja Palmunen for helping with the experiments and Alexander Frey for supervising.

I also want to thank you my family and friends who supported me through this time and gave me strength and motivation to keep working.

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Contents

1 Introduction

Spray drying is an important drying method in many fields of industry, including food, pharmaceutic and detergent industries. The history of spray drying goes back to 1872 when Samuel Percy patented a method to dry and concentrate liquid substances by atomization[1], now also known as spray drying. Some decades later, in 1920s, spray drying was first applied in the dairy and detergent industries[2,3] and in 1970s it became popular in instant coffee manufacturing[4]. Now, spray drying has a variety of new applications[3,5].

The great benefits of spray drying are that it is cost-effective compared to other drying methods like freeze-drying[6,7] and that the product stays at high temperatures only for a short period of time leading to only minor undesired reactions[4]. Therefore, spray drying suits heat-sensitive products too[4].

An example of a temperature sensitive product is an enzyme, neglecting several exceptions of enzymes that stay active in extreme conditions. At high temperatures enzymes start to degrade and deactivate, and that is why traditional methods like ovens do not offer an effective process. However, due to the speed of the spray drying, it can be used to produce enzyme powder from liquid enzyme solutions or suspensions[4,8].

Even though spray drying seems to solve the problem of drying enzymes without causing too much degradation and deactivation, another problem is faced with the enzyme powder produced. The smallest enzyme powder particles are so small, that they remain suspended in the air instead of settling on the floor where they can be cleaned by vacuuming. As enzymes are allergenic and can cause health issues for employees, lots of safety equipment is required. Separate rooms for enzyme powder manufacturing help to reduce the area where the full set of safety

equipment is necessary, but working in that area is complicated and needs education. If the smallest particles are removed from the product, the safety increases for all the employees as risk of exposure to enzymes is reduced.

The aim of this thesis is to study the effect of additives on the particle size and particle size distribution of enzymes in spray drying. Larger particle size along with increased agglomeration helps to reduce the most problematic enzyme dust and increase safety in enzyme powder manufacturing and research. Also, if the particle size can be controlled, the product would be more homogeneous, thus improving quality.

The Literature Part consists of three chapters: Spray drying, Particle formation, and Statistical analysis and hypothesis testing. The chapter on spray drying includes the different stages of the process, parameters and their effects, and applications. The second chapter describes the phenomena of particle formation and the methods to evaluate particles and their size distribution. The final chapter in the Literature Part concentrates on statistical analysis and hypothesis testing in order to explain the methods used to evaluate experimental data.

In the Experimental Part, spray drying of different products with several different additives is described, analyzed and evaluated using statistical analysis methods. Furthermore, the results from laboratory scale were compared to production scale results in order to see how well the laboratory scale models the production scale.

The literature published on this topic is mostly from the food and pharmaceutical industries. Especially in pharmaceuticals, the most common goal is to get smaller particles with special morphology to help medicines to be absorbed by the body. Studies on the effect additives have on the stability and morphology were available but no studies examining the effect on particle size was found.

LITERATURE PART

2 Spray drying

This chapter is about spray drying, in order to give better understanding on the unit operation for the reader. First, the general knowledge of spray drying is given. Second, the principle of the drying process and its different stages are explained. Third, the most important parameters and their effects on the product are described. Finally, some of the most common applications are introduced.

2.1 General

Spray drying is a unit operation used to transform solutions or suspensions into powders. It is a drying method where liquid is atomized and sprayed to a drying chamber. In the chamber the moisture is removed by high rate evaporation.[8-10]

Even though high temperatures are used, spray drying can be applied to temperature sensitive or unstable products without major losses of activity. Therefore, enzymes and other biological products can be dried by spray drying.[4] Another advantage in spray drying is that morphology of the particles can be controlled by the feed solution characteristics and drying parameters[11-13].

There are still challenges in spray drying. One major challenge is to optimize the process for the product, as small variations of the feed solution can cause large differences to the final product characteristics. Models and software have been created to help to plan and optimize spray drying processes[8,14-16].

2.2 Principle

The principle of the spray drying is simple. A feed solution is sprayed to a chamber as small droplets. Drying gas then heats up the droplets and causes evaporation and thus drying of the product.[4] In this section, the steps of spray drying are

explained in more detail starting from the feed and proceeding through atomization, evaporation and finishing with gas separation.

2.2.1 Feed

The feed solution has a high effect on the final product[5]. The particle size and shape and other characteristics can vary remarkably depending on the dry mass concentration, homogeneity, additives, or several other feed characteristics[4,5].

The feed solution must be pretreated so that it does not contain unwanted impurities or other components, as the drying process only removes the spare solvent or water. Also, to receive homogeneous product, feed solution should be homogeneous too.[4]

2.2.2 Additives

In order to get a product with desired properties, additives can be blended in the feed solution. They increase the dry mass concentration as well as help to control particle morphology and bulk density of the final product.[4] Examples of additives used in spray drying are salts like sodium chloride, polymers like maltodextrin or gelatin, and proteins[4,17].

Different additives are used for different purposes. Polymers like starches and maltodextrins are used as additives due their high molar masses[18,19]. They can be added to feed solution to increase the solids concentration, and thus improve the drying process (see Table 1 in Section 2.3 for more information). They are efficient in the cases where the product is amorphous with too low glass transition temperature causing stickiness and caking[19].

Another usage of additives is to precipitate the product. Salts can be used to precipitate proteins, like enzymes or antibodies[20,21]. An antibody IgG was

precipitated by ammonium carbamate in order to increase protein stability during spray drying[20]. Salts are also used to increase the solids concentration.

Additives are not always only helpful. The problem with maltodextrin, dextrin, NaCl and Na_2SO_4 was noted by Sloth *et al.* They found that each of those additives increased the droplet temperature. A problem occurred with higher additive concentrations when a strong skin was formed. Instead of evaporation through the pores of the skin, the inside of the droplet was gelatinized.[22]

Maltodextrin and dextrin were assumed to reduce the water activity by forming a film on the surface of the droplet and salts by changing the chemical potential of the droplet. As in high concentrations of these additives the resulting particles were gelatinized, Sloth *et al*. suggested the usage of these additives improper for temperature sensitive products.[22]

However, Gupta *et al*. proved that salts, polymers, sugars and sugar alcohols used in their study actually increased the stability of xylanase enzyme. The specific activity of the enzyme after spray drying was higher than without additives and the half-life was also increased. There were differences between the additives, and only several additives were chosen to be tested at different drying temperatures. Tests suggested that by choosing an appropriate temperature and additives 99% of activity could be retained through the spray drying process.[21]

2.2.3 Atomization

Atomization is the stage of drying where the droplets from the feed solution are produced. The nozzle of the spray drier has a high effect on the mean droplet size, and therefore on the particle characteristics of the product[4,13,23]. Four typically used nozzle types are described in this section: two fluid nozzle, rotary disk atomizer, pressure nozzle and ultrasonic nozzle (Figure 1).

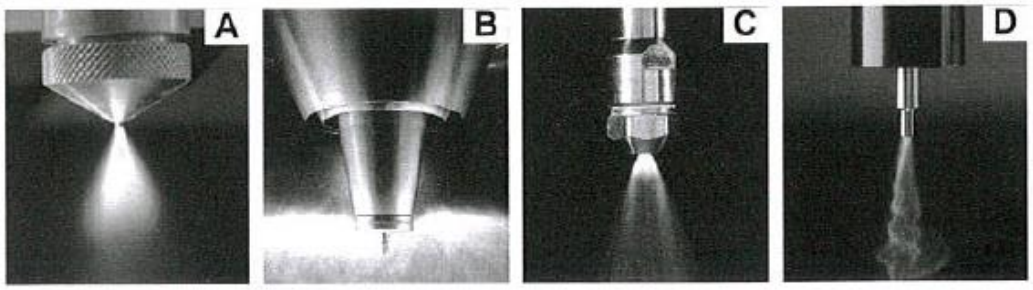

Figure 1. *Different types of nozzles for spray drying exist and the spray produced depends on the nozzle characteristics. A) Two fluid nozzle B) Rotary disk atomizer C) Pressure nozzle D) Ultrasonic nozzle.*[4]

Two fluid nozzles are commonly used in research[6,16,18,21]. The energy for atomization is provided by the spray gas mixed with the feed solution. When the gas-liquid mixture enters the drying chamber, the gas expands rapidly and produces droplets. The droplet size can be controlled by the gas-liquid mass ratio: higher gas ratio (>3:1) results smaller droplets (5-20 microns) and smaller ratio (0.5:1) larger droplets (125 microns).[4]

Rotary disk atomizers spray the feed solutions into the drying chamber as a fine mist that is dried by a concurrently flowing gas. The benefit of the rotary disks is that they provide high feed throughput, which makes them applicable in industrial scale. The droplet size is controlled by the peripheral velocity of the disks, where higher velocity (>180 m/s) causes smaller droplets (20-40 microns) and lower velocity (75 m/s) larger droplets (225 microns).[4]

Pressure nozzles are used if large droplets are wanted. The pressure provided creates a thin film of the feed which enables drying of larger particles. The particle size depends on the feed rate and viscosity, but the mean particle size is controlled by the pressure. Lower pressure, around 15-25 bars creates larger droplets in the range of 150-350 microns while over a 100 bar pressure creates droplets of around 20-40 microns.[4]

Ultrasonic nozzles control the droplet size by frequency of the nozzle vibration. Surface tension and viscosity of the feed solution also have an effect on droplet size.[4] This technique gives highly uniform droplets, which makes it important in the research of particle size and morphology[12]. However, the largest droplets produced by the ultrasonic nozzle are only around 70 microns (25 kHz) and smallest 18 microns (120 kHz), which is relatively narrow range compared to the others nozzles introduced.[4]

2.2.4 Evaporation

When liquid droplets come into contact with the spray gas, evaporation begins on the droplet surface immediately[4]. The spray gas can be air, an inert gas like nitrogen or argon, or steam[4,13]. Due to the high surface area of small droplets both heat and mass transfer are intensive and result an efficient drying[4,8]. The evaporation phenomenon is explained in more detail in Section 3.2.

To understand the evaporation stage, basic knowledge on evaporation is required. Phase change from liquid droplet to gas requires energy and that energy is taken from the spray gas. When biological products are spray dried, it is important to work at low temperatures (inlet temperature 145-160 °C, outlet temperature <80 °C)[10,24]. Inlet temperature cannot be decreased too much as the moisture content of the product has to stay low (<6 %). By increasing the feed rate, outlet temperature decreases as more energy from the spray gas is required to evaporation of solvent from droplets. Reduced energy in spray gas causes decreased outlet temperature. More about parameters and their effects in spray drying is described in Section 2.3.

2.2.5 Gas separation

Before the final product is received, the gas must be separated. Examples of separators are a cyclone (Figure 2)[4], filter bag and electrostatic precipitator[3]. In the cyclone, the solid particles flow to the bottom of the device where a collection

dish is connected and the gas flows out from top. In order to ensure that no solid particles stay in the gas, a filter can be used after the cyclone.

Figure 2. *A cyclone (front) can be used to separate gas and solid product in spray drying. To ensure solid-free gas release, a filter can be connected in series after cyclone (back).*[4]

2.3 Parameters and their effects on the product

An important part of spray drying is to optimize the process. In order to do so, the effects of drying parameters on the product must be well understood. In Table 1, the parameters for Mini Spray Dryer (more information on the device in Table 4) and their effects on the product are described by the manufacturers[4]. As can be noticed, the strength of the effect varies from no effect (or insignificantly small effect) to very strong effect.

Device manufacturers often offer some estimation of the optimal conditions on different products. Using the offered initial values together with a table like to Table 1, a reasonable guess on the conditions can be obtained. Still, the final optimization is usually done by using a “Trial and Error” procedure.

For heat sensitive products like enzymes and other biotechnological products, the temperature is one of the most important variables. In order to avoid deactivation and degradation of the product the parameters affecting it must be controlled. The most effective way to control outlet temperature is to control inlet temperature (Table 1).

Table 1. *The effect of increase in aspirator rate, air humidity, inlet temperature, air flow, feed rate, solvent and feed concentration on the outlet temperature, particle size, final humidity of the product and yield is described by arrows showing the strength (the number of arrows) and the direction of effect with Mini Spray Dryer B-290.*[4]

Parameter/ Dependance	Aspirator rate ↑	Air humidity ↑	Inlet temperature ↑	Spray air flow ↑	Feed rate ↑	Solvent instead of water	Feed concentration ↑
Outlet temperature	↑↑ Less heat losses baded on total inlet of energy	↑ More energy stored in humidity	↑↑↑ Direct proportion	↓ More cool air to be heated up	↓↓ More solvent to evaporate	↑↑↑ Less heat of energy of solvent	↑↑ Less water to be evaporated
Particle size	-	-	-	↓↓↓ More energy for fluid dispersion	(↑)More fluid to disperse	(↓)Less surface tension	↑↑↑ More remaining product
Final humidity of the product	↑↑ Lower partial pressure of evaporated water	↑↑ Higher partial pressure of drying air	↓↓ Lower relative humidity in air	-	↑↑ More water leads to higher particle pressure	↓↓↓ No water in feed leads to very dry product	↓ Less evaporated, lower partial pressure
Yield	↑↑ Better separation rate in cyclone	(↓)More humidity can lead to sticking product	(↑)Eventually dryer product prevent sticking	-	(↑↓)Depends on application	↑↑ No hydroscopic behavious leads to easier drying	↑ Bigger particles lead to higher separation

2.4 Applications

Spray drying is a widely used drying method in several fields of industry. In this section some of its most common applications in the product formulation in biotechnological, food and pharmaceutical industries are introduced as well as several others including detergents and nanoparticles.

2.4.1 Enzymes and other biological products

Drying is an efficient method to increase shelf-life of biological products like enzymes and other proteins[25]. There are several drying methods available, but spray drying enables efficient drying without major losses in activity if the parameters are correctly chosen.[4,24]

For efficient drying with high enzyme activity preservation the choice of drying parameters is important[10,24]. The preservation of enzyme activity is of course important as generally enzymes and other biological products are produced because of their biological activity.

In addition to drying parameters, use of additives can help to stabilize the product. There are significant differences in results between studies of different enzymes and other biological products.[21,26,27] For example, Selianov noticed that some common salts were not effective stabilizers for cellulases, but instead, different forms of cellulose were[26]. In contrast, Gupta found out that many salts are effective stabilizers for xylose, and that using salts together with polyols or polymers, the effect can be enhanced remarkably[21].

One reason for the differences in the stabilizing effects is the differences in the additive function. Salts can be used to precipitate the enzyme and that way protect it during spray drying[20]. Maltodextrin can be used to immobilize the enzyme, and

sugars like trehalose to encapsulate, and thus, protect the enzyme[21,28,29]. In some cases, polyols like sorbitol are used to avoid aggregation[27].

In order to use proper drying parameters and additives for the products, the stabilizing effects of different additives on the enzyme or other biological products must be understood. There are some publications available, but only with the most common products and additives.[10,21,24,26,27]

2.4.2 Food industry

In 1920s, milk powder was produced for the first time. It was the first time spray drying was used in food industry, and together with detergent industry, the first commercial application of spray drying[2,3].

Milk is dried to increase its shelf-life and to reduce transportation and storage costs. Also, milk powder is easy to use in food industries that use milk as raw material.[16,30] An important factor to evaluate the milk powder quality is the moisture content, and there are standards for the highest acceptable moisture levels[16].

Currently, spray drying is used for several food products including egg products, beverages, vegetable proteins, fruit and vegetable extracts, carbohydrates, tea extracts and yogurt, among others[3,17,18,31,32].

2.4.3 Pharmaceutical industry

Spray drying has become of great interest in the pharmaceutical industry. The increased control of morphology and particle size enables the production of new formulation of drugs. For example, oral drugs could be replaced by inhalable drugs as they often require lower dosage.[33-35]

In many cases, carriers are used to transport the drug. By using additives and controlling the parameters of spray drying, the morphology of the drug can be modified so that carriers are no longer necessary[33]. This way long storage times will not affect the adhesion characteristics between the drug and carrier, and therefore the effectiveness of the drug[36].

Even though many products are still at the theoretical or experimental stage, there are already several pharmaceuticals produced using spray drying[34].

2.4.4 Others

In addition to biological products, food and pharmaceutical industries, there are many other applications for spray drying. In this section, only detergents and nanoparticles are introduced, but spray drying is used in chemical industry in production of catalysts, dyes and several other products[5].

Detergents were produced by spray drying already in 1920s[2,3]. As the technology is widely used, there are not many recent studies on topic available. Furthermore, some standardized systems have been developed and used all over the world.[37]

Nanoparticles can also be produced by spray drying. The advantage of using spray drying is that higher concentrations of reacting species can be used compared to other methods like precipitation methods or reversed micelles.[5] There are several studies on how to produce desired kind of particles by spray drying, as in nanotechnology, the morphology and particle size distribution are important for the applications. Nanoparticles can be used in electronics, optics, sensors, and drugs, among others.[5,11-13,23,38]

3 Particle formation

In this chapter, the particle formation is discussed in greater detail. First, general information on particle formation is given. Second, the phenomena behind the particle formation from droplet, and interactions are explained. Third, the particle morphology is introduced in order to better understand the drying process, and finally the particle size distribution and how to measure it is explained.

3.1 General

Solid particles form during the evaporation stage of the spray drying (Section 2.2.4)[4]. They differ in many ways including size, moisture content and shape, but also by their homogeneity, charge, bulk density and ability to agglomerate[4,39].

Depending on the particle application, different characteristics are important. In case of enzymes and other biomaterials, it is important that the final product is still active and that the moisture content is low enough to improve storage life[25].

3.2 Phenomena

Droplet transformation into a particle is a widely studied phenomenon, starting in the 1950s, when Ranz and Marshall first described evaporation from droplet. In 2015, Mezhericher *et al*. reviewed all the stages of drying, in order to create a software to simulate and model spray drying.[8,38,40]

Figure 3 shows the basic idea of droplet transformation into a particle in the spray drying process. First, the droplet is heated to its evaporation temperature followed by evaporation of the surface layer. Second, a skin is formed around the droplet transforming it to a wet particle. Last, the wet particle dries and is heated to surrounding temperature.

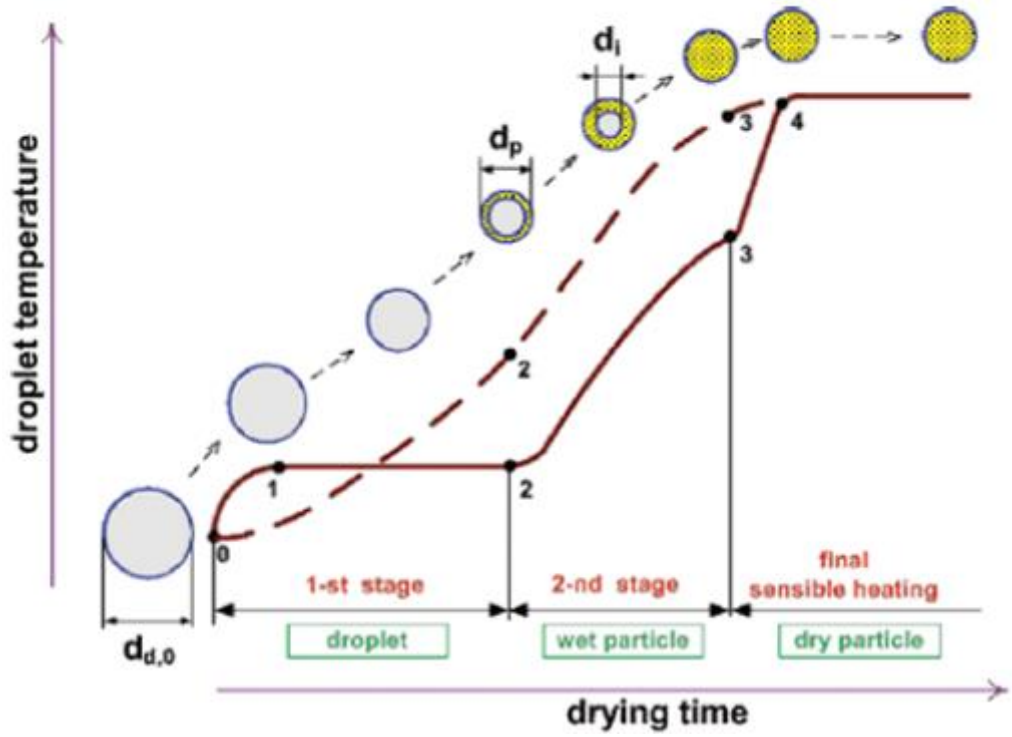

Figure 3. *Droplet temperature varies at different stages of drying. Solid line: suspension, dash line: solution*[8]

Temperature of the droplet varies during the drying and it depends on the feed solution characteristics. For solutions, temperature rises quite smoothly during the whole drying time, whereas for suspensions clear steps for initial heating, evaporation and particle drying can be seen (Figure 3).[8] This is due to differences in evaporation process. Dissolved materials like salts change the evaporation temperature of the solution compared to pure water. When the water evaporates, the salt concentration increases and causes the evaporation temperature to increase too. This is why there is no stage with a constant temperature.

Unlike solutions, suspensions consist of two or more phases. One phase does not affect the evaporation temperature of the other phase, so when the temperature

reaches the evaporation temperature of water, it stays constant until the skin is formed which starts to disturb the evaporation.[41]

3.2.1 Initial heating and evaporation

The first step in particle formation is the initial heating of the droplet to its equilibrium evaporation temperature. The equilibrium evaporation temperature is defined where the droplet surface temperature stays constant.[8] In this equilibrium state, the energy provided by the drying gas and the energy needed for the evaporation from the droplet surface are equal.

However, depending on the droplet characteristics, the temperature might not have an equilibrium evaporation temperature. Thus, different equations and models apply to solutions and suspensions[8,38]. However, in the simplest case the evaporation happens on the droplet surface, the droplet shrinks and gets more concentrated[8]. Eventually, the solid concentration on the surface is so high that a skin, or crust is formed[8,39].

In most cases, the process is more complicated and many variables have to be included. Some factors like density and specific heat capacity are functions of temperature. Especially during the initial heating and evaporation, when the temperature is not constant, this temperature dependence has to be taken into account. Because the equations are complicated, technology can be a great help in calculations or modeling of process.[8]

3.2.2 Particle formation and final drying

Particle formation starts in the end of evaporation stage, when a skin around the droplet is formed. Then the droplet is called a wet particle. However, the transformation is not always immediately completed after skin formation.[8] The initial skin might not be strong enough and collapses due to compressive capillary forces caused by the higher pressure outside of the droplet[14]. Then the

evaporation continues until a new skin is formed. This may happen several times before the final, stable skin is formed. This stage is called transition period.[8,14]

According to Mezhericher *et al.* the particle drying happens by vapor diffusion through the pores in the skin[8]. The temperature of the particle increases as the moisture content decreases until the particle is dry and reaches the temperature of the environment (Figure 3).

3.2.3 Interactions

Although the studies on the particle formation increase the understanding of the drying phenomena, often systems with only one droplet or particle are studied[8,9,40]. However, the spray drying process involves a great number of droplets and particles that interact.

Droplets interact by droplet-droplet collisions. The type of collision can be a bounce, coalescence or breakage of the colliding droplets. A bounce causes the change of velocity and direction of the droplet. This can affect the evaporation from the droplet surface.[8]

Droplets may combine and form larger droplets, or break into smaller droplets by collision.[8] The change in droplet diameter has a significant effect on the drying process making it faster or slower. Smaller droplets dry faster than larger droplets due to larger surface to volume ratio, and the rate of drying affects the morphology, among other characteristics[8,39].

In addition to droplets, particles can also interact by collisions[8,39]. The type of collision depends on the particles, as hard-spheres are more probably bouncing than breaking up, but hollow particles may break. Also the moisture content of the particles has an effect on the interactions as wet particles may combine and cause agglomerates[39].

In the spray drying process, droplets and particles also collide with the walls of the drying chamber[8]. Those collisions can cause chamber fouling, and thus, decrease the yield[42]. Dry and crystalline particles do not stick to chamber wall as easily as wet particles, and droplet-wall collisions can be reduced by adjusting the nozzle so that the feed does not touch the walls before it dries[42,43].

3.3 Particle morphology

Particle morphology describes the shape of the particle. The shape can be dense spherical, hollow, doughnut shaped, encapsulated, hairy or porous, among others (Figure 4). There are several studies on particle morphology in spray drying on how to receive certain type of particles[5,13,23,39]. As the nanoparticle morphology plays an important role in many applications, like drug carriers in the pharmaceutical industry and superconductors in electronics, the development of methods to produce particles with desired morphology are of great interest[5,23].

From Figure 4 it can be noticed that the particle types can be divided into two groups. There are particles formed of one component only and particles formed of two components (composite particles). Some of the structures are formed in several steps like the formation of porous particles.[13]

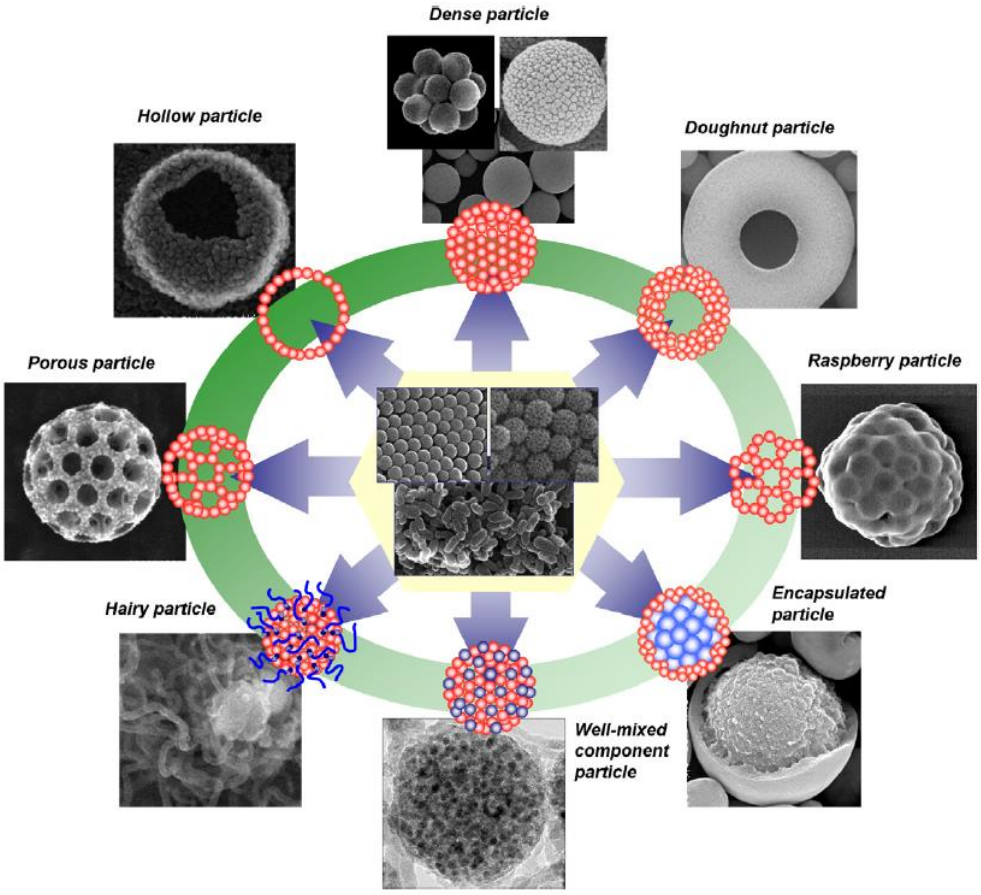

Figure 4. *Particles from spray drying can have different morphologies depending on how they were dried.*[13]

When a solution with only one component is spray dried, dense, hollow or doughnut particles can be formed from the same solution by varying the drying parameters[13,44]. When the evaporation rate of a droplet is low the result is a dense particle, whereas with high evaporation rates the particle is hollow[44]. If the drying gas flow or the drying temperature in the chamber is increased, particles are destabilized and change morphology from spherical to "mushroom hat" towards the doughnut shape[11]. An example of a single component solution is an inorganic solution used in the production of nanoparticles[13].

Two-component solutions form either encapsulated or well-mixed particles depending on the particle size difference of the two components. If they are of equal size, well-mixed particles are formed.[13]

Depending on the particle characteristics or state of drying (i.e. the moisture content), agglomeration may occur. The main reasons for agglomeration according to Walton and Mumford are the moisture content and static electrical effects[39].

3.4 Particle size distribution

The product of spray drying is not homogenous, but instead, the particles vary in size[4]. The feed solution properties and drying conditions have an effect on the final particle size distribution, and by understanding the effects the distribution can be modified into a desired direction[24,45,46].

Narrow distribution means that the product is close to homogeneous in particle size. This is a desired characteristic in many fields of study and industry as the applications often require homogeneity in order to create products with high quality[5,23,44].

Particle size distribution can be measured by devices designed for that. Several different methods are used for the detection of the particle size depending on the size range[47]. A common method with a wide size range is laser diffraction. Other methods include dynamic light scattering, resonant mass measurement, and Raman or imaging[47,48].

4 Statistical analysis and hypothesis testing

In the evaluation of experimental data and its reliability, statistical analysis and hypothesis testing are useful tools. This chapter introduces the most important statistic values and several commonly used hypothesis testing methods.

4.1 General

Statistical analysis is based on the collected data and its statistics. Some of the most important statistics are means and variances, along with standard deviation and covariance. With the help of estimators, the experimental data can be used to evaluate the hypothesis. There exist several ways to execute hypothesis testing, of which *t*-test and χ^2-test can be used in a variety of applications.[49,50]

Also, several ways to evaluate the data by hypothesis testing exist. Hypothesis testing is based on critical values and rejection area or the *p*-value. Those values help to either accept or reject the hypothesis.[49]

4.1.1 Statistic values

While there are several different types of means and variances, the most common to this work are the arithmetic mean, $\bar{X}$ or $\bar{x}$, and the sample variance, s^2. The equation for $\bar{x}$ is described below in the Equation 1, and Equation 2 describes the sample variance:

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i \tag{1}$$

$$s^2 = \frac{1}{n-1}\sum_{i=1}^{n} (x_i - \bar{x})^2 \tag{2}$$

where n is the number of samples, x_i is the value of ith sample.[49]

The expected value, E(X), is the value of the discrete random variable X that is most probably the mean of X. The variance of X is sometimes denoted as Var(X). This notation helps to differentiate variances of different variables as variance can be calculated to arithmetic mean or other estimators. An expected value denoted as μ refers to an expected value using normally distributed density function N and σ^2 to the variance of N. [49,51]

Standard deviation is the square root of variance, denoted as σ or s_x. Standard deviation of the sample means is also called the standard error of the estimate. [49,50]

Variance is a special case of covariance. Covariance describes how two different random variables change together. That information can be used in the evaluation of regression lines to test linear correlation between plotted data points. Equation 3 is used to calculate the covariance.

$$S_{xy} = \frac{1}{n}\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y}) \quad (3)$$

where n is the number of samples, x_i and y_i are the values of ith samples and $\bar{x}$ and $\bar{y}$ are the arithmetic means. [50]

4.1.2 Null and alternative hypothesis

Hypothesis testing can be used to evaluate data and is widely applied in statistical analysis. In order to understand different tests introduced in Sections 4.2 and 4.3, some basics of hypothesis testing should be explained.

Hypothesis testing starts with a general hypothesis often denoted as H. H defines the variables of the test. After that a null hypothesis is defined and denoted as H_0. In its simplest form H_0 can be defined as "equals". For example with a discrete density function $f(x;\theta)$, where x is a data point and θ is an unknown parameter, null hypothesis can simply have a form

$$H_0: \theta = \theta_0 \quad (4)$$

where θ_0 is the expected value of the parameter θ according to the H_0. Hypothesis testing can be used to evaluate calibration ($H_0: \mu = \mu_0$, where μ_0 is the expected mean) or correlation (Section 4.5), to mention a few applications. In the case that H_0 does not apply, the alternative hypothesis H_1 comes into effect. H_1 is also defined before the actual hypothesis testing. In the case of Equation 4, H_1 can be defined according to Equations 5, 6 and 7. [49]

$$H_1: \theta < \theta_0 \quad (5)$$

$$H_1: \theta > \theta_0 \quad (6)$$

$$H_1: \theta \neq \theta_0 \quad (7)$$

4.1.3 Critical values and rejection area

After defining a null and an alternative hypothesis a rejection area must be defined. A significance level α is chosen and is a probability for a test variable to be in the rejection area. Common significance levels are 0.05, 0.01 and 0.001. Using statistical tables, critical values for the test variables can be found. [49,52]

As an example for finding the rejection area, the hypotheses defined in Equations 4 and 5 are used. For Equation 5 the rejection area for test variable distribution Z is defined by the following Equation 8:

$$\Pr(Z \leq l|\mathrm{H}_0) = \alpha \tag{8}$$

where *l* is the critical value for the test variable. See the Figure 5 for a graphical representation. [49]

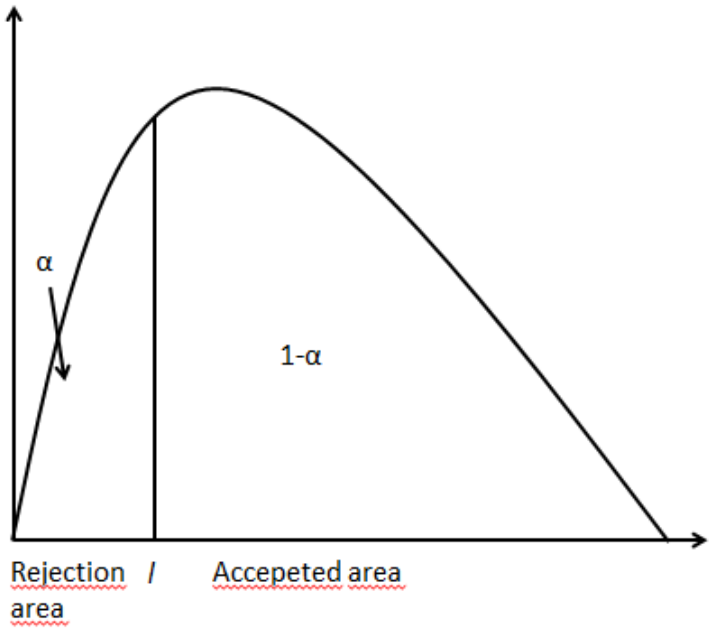

Figure 5. *Test variable density function with a significance level α and a critical value l. The values equal to or smaller than l are in the rejection area and the null hypothesis does not apply.*

4.1.4 *p*-value

Another way to make decisions whether to accept the null hypothesis is to use *p*-values. The *p*-value is a probability that a test variable gets an improbable value even if the null hypothesis applies. A small *p*-value is defined and denoted as p_0. Using statistical tables, the *p*-value for the test variable can be calculated. If *p* is smaller than p_0, then the null hypothesis most probably does not apply and the alternative hypothesis comes into effect. The following Equations 9, 10 and 11 can be used to calculate p-values for the hypotheses used previously in Equations 5, 6 and 7, respectively.

$$p = \Pr(Z \leq z|\mathrm{H}_0)\ , \tag{9}$$

$$p = \Pr(Z \geq z|\mathrm{H}_0) \text{ and} \tag{10}$$

$$2p = \Pr(Z \geq |z||\mathrm{H}_0)\ . \tag{11}$$

where z is the test variable calculated from the data and H_0 is the null hypothesis from Equation 4. [49]

4.2 *t*-test

t-test is a commonly used test for hypothesis testing for an expected value. It can be used to test one random variable or to simultaneously test two unrelated random variables for agreement with the hypothesis. Additionally the *t*-test can be used to compare values of pairs.[49]

For perferming *t*-test, several assumptions are made. The mean of the collected data is assumed to follow a normal distribution density function *N* with known μ and σ^2 [50]. For evaluation of one or two random variables, sample mean $\bar{X}$ and sample variance s^2 must be calculated using Equations 1 and 2. Then the *t*-value can be calculated. For one random variable,

$$t = \frac{\bar{X} - \mu_0}{\frac{s}{\sqrt{n}}} \tag{12}$$

where $\bar{X}$ is the sample mean, μ_0 is the expected value, s is the standard deviation and n is the sample size. There are $(n - 1)$ degrees of freedom.[49,50] The number of degrees of freedom is the number of values that can vary without affecting the system. Often the degrees of freedom are defined as the number of data points subtracted by the number of parameters.[53]

The rejection area and critical values can be calculated as in Section 4.1.3 and then *t*-value calculated from Equation 12 can be compared to critical values received from statistical tables.[49,52]

For two random variables the t-value can be calculated using either Equation 13 or Equation 14 depending on the variance. If variances of random variables are different, Equation 13 is used.[49]

$$t_A = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \tag{13}$$

If variances of random variables are the same, Equation 14 is used.[49]

$$t_B = \frac{\bar{X}_1 - \bar{X}_2}{s_p\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \tag{14}$$

where s_p is a pooled variance:

$$s_p = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \tag{15}$$

Degrees of freedom are easy to define for t_B as it is simply $(n_1 + n_2 - 2)$, but for t_A approximations must be used. One example is the Satterthwaiten approximation (Equation 16) which is rounded down to the closest full number.[49]

$$v = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)^2}{\frac{1}{n_1 - 1}\left(\frac{s_1^2}{n_1}\right)^2 + \frac{1}{n_2 - 1}\left(\frac{s_2^2}{n_2}\right)^2} \tag{16}$$

Pair comparison is used for data where experimental data is formed from pairs and it can be used if there are two identical experiments done, among other cases. The difference between data pairs (D_i) is used for the calculations. The sample mean, sample variance and *t*-value are calculated using Equations 17–20.[49]

$$D_i = \bar{X}_1 - \bar{X}_2 \tag{17}$$

$$\overline{D} = \frac{1}{n}\sum_{i=1}^{n} D_i \tag{18}$$

$$s_D^2 = \frac{1}{n-1}\sum_{i=1}^{n} (D_i - \overline{D})^2 \tag{19}$$

$$t = \frac{\overline{D}}{\frac{s_D}{\sqrt{n}}} \tag{20}$$

4.3 χ^2-test

χ^2-test is a commonly used test when the expected value μ is unknown but the variance is known for a random variable.[49] The test begins with defining the hypotheses.

General hypothesis H is that discrete random variable *X* has μ and σ^2 following the normal distribution *N*, i.e., $X_i \sim N(\mu, \sigma^2)$. The null hypothesis H_0 is

$$\mathrm{H}_0 : \sigma^2 = \sigma_0^2 \tag{21}$$

where σ_0^2 is the expected variance.[49]

Arithmetic mean and sample variance for *X* can be calculated using Equations 1 and 2. Knowing them, a test variable χ^2 can be calculated using Equation 22.

$$\chi^2 = \frac{(n-1)s^2}{\sigma_0^2} \tag{22}$$

where $(n-1)$ are the degrees of freedom. If H_0 is valid,

$$E(\chi^2) = n - 1 \tag{23}$$

This means that both large and small values of χ^2 suggest that null hypothesis should be rejected. The rejection area and *p*-value can be found similarly as described in Sections 4.1.3 and 4.1.4.[49,50]

4.4 Regression analysis

Regression analysis is the most commonly used and applied method in statistical analysis. It concentrates on explaining the relationship between variables of measured data. Regression analysis explains the strength of the relationship as well as helps to predict the results of further experiments, and can be used to create a regression model.[49]

Regression models can be divided into linear and nonlinear functions. Evaluation of nonlinear models is challenging, so it is recommended to linearize the model and use regression analysis for the linearized model.[49,50] A linearized model can either be a deterministic model or regression function. They have similarities but they are based on different ways to treat the data.

A deterministic model is used to define the relationship between the variables using observations by looking for a model that best fits to data points. It is often in the form of Equation 25. A regression function (Equation 26) is the conditional expected value of *y* as a function of conditional variable *x*. So the function is defined so that the expected value of *y* can be predicted as well as possible using values of *x*.

$$y = f(x\,;\,\beta) \tag{24}$$

$$E(y|x) = f(x\,;\,\beta) \tag{25}$$

where *y* is a variable, *x* is the determining variable and β is an unknown parameter that determines the shape of the function *f*. If a value for β can be found and it is always constant, the function is fully determined. This is, however, rarely the situation. In order to solve the problem, the equations are rewritten into the form of Equation 26.[49]

$$y_i = f(x_i\,;\,\beta) + \varepsilon_i \tag{26}$$

where ε_i is the residual term varying in each observation unit. To get the best fitting function for the model, β should be chosen so that ε_i is as small as possible for each observation.[49] One method to solve the value of β is to use the least squares method described in Section 4.4.1.[49,50]

4.4.1 Least squares method

Least squares method is a method to solve regression problems mentioned in Section 4.4.[49,50] It is based on finding the smallest square sum of residual term ε_i of a linear regression function in respect to regression factors $\beta_0, \beta_1, \dots, \beta_k$.

$$\sum_{i=1}^{n} \varepsilon_i^2 = \sum_{i=1}^{n} (y_i - \beta_0 - \beta_1 x_{i1} - \beta_2 x_{i2} - \cdots - \beta_k x_{ik})^2 \tag{27}$$

The minimal value of $\sum_{i=1}^{n} \varepsilon_i^2$ in Equation 27 is generally done by derivation. The solutions for $\beta_0, \beta_1, \dots, \beta_k$ are estimators often denoted as $b_0, b_1, \dots, b_k$.[49] Different computer programs can be used to calculate the estimators and find the regression function, and therefore help to evaluate the data.

4.5 Correlation

Correlation describes the statistical dependence of two variables. For linear correlation, dependence can be evaluated by using correlation coefficient, r_{xy}. The equation for the calculation of the correlation coeffiecient is described below in Equation 28.[49,50]

$$r_{xy} = \frac{S_{xy}}{S_x S_y} \tag{28}$$

where S_{xy} is the covariance of *x* and *y* of the data according to Equation 3, S_x and S_y are the standard deviations of standard variances of x and *y* of data according to Equation 2.

The values of r_{xy} vary between ±1, where positive value means positive slope of regression line and vice versa. Values of $|r_{xy}|$ close to 1 mean strong linear dependence of the data points, and values close to 0 mean no linear correlation. However, that does not mean no dependence as the dependence can be nonlinear.[49,50]

The correlation coefficient can also be used for nonlinear dependence, but only if the dependence can be linearized by axis transformation.[50]

For evaluation of correlation, *t*-test in Section 4.2 can be used. One way is to set the null hypothesis

$$\mathrm{H}_0 : \rho_{xy} = 0 \tag{29}$$

where ρ_{xy} is the correlation of the variables *x* and *y*. In this case there exists no linear correlation if H_0 applies.[49]

EXPERIMENTAL PART

5 Aim

The aim of this study was to study the effect of additives on enzyme particle size in spray drying and removing the smallest particles (diameter <10 µm) using particle size distribution analyzer and SEM to determine the particle size. The chosen additives were salts like sodium chloride, sodium sulphate, calcium acetate and calcium chloride, and methyl cellulose. Their effect on particle size was studied using three different enzyme solutions.

The three enzyme solutions were chosen so that the differences between the effects of additives on different enzymes could be compared.

Statistical analysis methods were used to analyze the statistical reliability of the resulting data, correlations and conclusions drawn from them.

6 Materials and methods

In this chapter, the materials, equipment and methods used in the study are described.

6.1 Materials

The enzyme solutions used are named as 1A and 1B, 2, and 3 where numbering refers to different enzymes, and A and B to same enzyme produced by different strains (Table 2). The samples of enzyme 3 consisted of three different batches, one from production scale and two from pilot scale. They were named as 3.1, 3.2 and 3.3, respectively.

Table 2. *Enzyme solutions were named by numbering and different strains were defined by A and B.*

Enzyme solution name	Explanation
1A	Enzyme 1, strain A
1B	Enzyme 1, strain B
2	Enzyme 2
3A	Enzyme 3, production scale with media 1
3B	Enzyme 3, pilot scale with media 1
3C	Enzyme 3, pilot scale with media 2

Enzyme solutions were received as concentrates and to get lower concentrations, filtrate of enzyme solution was used to dilute.

Different additives were mixed then with the enzyme solutions. The information on the additives used is listed in Table 3.

Table 3. *Information on substances used includes producer and producer country.*

Substance name	Producer	Production country
Ensure® Sodium Chloride for analysis	Merck KGaA	Denmark
Sodium sulphite without water	-	-
Methyl cellulose, 400 cP	Sigma-Aldrich	USA
Sodium hydroxide, 4 M		
Calcium acetate hydrate	Merck	Germany
Calcium chloride dihydrate cryst. GR for analysis	Merck KGaA	Germany
L(+)-Ascorbic acid	VWB Chemicals	Belgium

6.2 Equipment

Equipment used in the study included the devices in Table 4 along with basic laboratory equipment.

Table 4. *Information on devices includes producer, serial number and producer country.*

Device name	Producer	Serial number	Producer country
MB23 Dry weight analyzer	OHAUS CORPORATION	25165166	China
BÜCHI Mini Spray Dryer B-290	BÜCHI Labortechnik AG	1000142364	Switzerland
Beckman Coulter™ LS 13 320 Laser Diffraction Particle Size Analyzer	Beckman Coulter™	AE 27316	USA

6.3 Methods

Methods used in this work include sample preparations, spray drying and different analysis methods.

6.3.1 Preparations

Enzyme solutions were stored in a cold room (T = 6 °C). Additives were weighed and mixed with the solutions to get 200 grams (wet weight) of sample in total.

The dry weight percentages of enzyme solution concentrates 1A, 1B and 2 were between 20 and 25 % and between 10 and 15 of enzyme 3. The concentrations of NaCl were 5, 6.8, 7.5, 12.5, 15, 17.5 and 20 w/w % (grams of additive per grams of enzyme solution). For Na_2SO_4 the concentrations were 4, 5, 6 and 6.8 w/w %. Ca-

acetate and $CaCl_2$ were at 2 w/w % concentration, methyl cellulose at 1.5 w/w %, and ascorbic acid at 0.5 w/w %.

If the enzyme solutions were diluted, it was done 1:1 (concentrate : filtrate) and named as mix. Thus, the concentrations of the enzyme solutions can be defined as concentration factors. For filtrate the concentration factor is 1, for mix it is $\frac{1+X}{2}$, and for concentrate it is X. If for example the concentration factor for the concentrate is 2, then for the mix it is 1.5.

The pH of several samples was adjusted and it was performed after addition of other additives using 4 M NaOH solution.

If the sample was not spray dried immediately, it was stored in the cold room. Also if the additive did not dissolve easily or precipitated so that mixing would not make the solution homogeneous again after storage, solution was stored under mixing.

6.3.2 Spray drying

Before drying, the spray dryer (Table 4) was prepared and the drying parameters were set. The parameters used depended on the type of cyclone used. All experiments except for enzyme 1A were done with the large (standard) cyclone. The used parameters are collected in Table 5.

Table 5. *Parameters of spray drying differ for large (standard) and small (high-performance) cyclone.*

Parameter	Large cyclone	Small cyclone
Aspirator (%)	100	75
Underpressure in the system (mbar)	60	60
Spraying pressure (mm)	40	40
Inlet temperature (°C)*	140	160
Pump speed (%)	30	15
Outlet temperature (°C)	75	75
Nozzle tip size (mm)	0.7	0.7
Nozzle cap size (mm)	140/150	140/150

*The inlet temperature was the parameter used to adjust outlet temperature to stay at 75 °C, so the value given here is the value used in the beginning of the drying before adjustments.

Inlet temperature was the parameter used to adjust outlet temperature to 75 °C, as the effect of inlet temperature on the particle size has the least effect according to Table 1 (Section 2.3).

First the spray drier was run with deionized water. When the system was stable (outlet temperature did not change) and the outlet temperature was checked to be 75 °C, the sample was run. The outlet temperature was kept constant by adjusting the inlet temperature. When the drying was finished, powder samples were stored at room temperature until analyses and liquid samples of starting material were kept (approximately 10 g) in the freezer (−21 °C).

Different experiments were done for different enzymes in order to find the optimal drying additive, dry weight and ratio between enzyme and additive. For enzyme 1A the aim was to study differences between the two cyclones and nozzle caps to get as large particles as possible. For enzyme 1B the aim was to study the

effect of enzyme solution concentration and additive concentration with NaCl and Na_2SO_4.

For enzyme 2 the NaCl concentration and solution pH was varied and combined with methyl cellulose. Furthermore, the effect of Na_2SO_4 was studied at constant concentration.

Enzyme 3 was studied at constant enzyme concentration but varying NaCl concentration and pH along with the effect of Ca-acetate, $CaCl_2$, methyl cellulose and ascorbic acid. Also enzyme solutions from different scales and media were compared. All the experiments can be found from the Chapter 7 before each section containing the results.

6.3.3 Analysis

Several different analyses were done for the samples. Dry weight content as well as enzyme activity were analyzed of the liquid samples and dried products. pH of the liquid sample was measured before drying. For the solid sample, particle size distribution was examined using particle size analyzer (Table 4). An overview of the used sample analyses and measurements is provided in Table 6.

Table 6. *Overview of different analyses done for the liquid and the solid samples.*

Analysis/measurement	Liquid sample	Powder sample
pH	x	
Dry weight (%)	x	x
Activity (U/g)	x	x
Particle size distribution		x
Microscope pictures	x* (optical)	x* (SEM)
Element analysis		x*

*Chosen samples

The dry weight of the samples was measured by adding approximately 1 g of the sample to MB23 Dry weight analyzer (Table 4). The device was set to measure the difference in weight after 10 minutes of heating at 105 °C.

Specific enzyme activity was measured by the Quality Control laboratory of Roal Oy. Samples from the feed solution and powder were analyzed in order to receive the initial and final specific activity in units of enzyme per grams of enzyme sample. The theoretical specific activity was calculated by using dry weights of the samples and the yield was calculated from the difference between the theoretical specific activity and analyzed specific activity of the powder.

Beckman Coulter^{TM} LS I3 320 Laser Diffraction Particle Size Analyzer (Table 4) was used to analyze the particle size and particle size distribution. Approximately 3-4 cm^3 of powder was used for the analysis. Before the measurements, the device was set to align and measure offset points and background. Then the actual measurement was done. The background was re-measured before testing each sample.

The particle size analyzer gave results as distribution curves of which statistics like mean and mode were calculated by the program running the analyzer. It was chosen to study the effect of the particle size by the mean particle size and D(3,2) value. D(3,2) is the surface weighed mean diameter of the particles.

For the liquid samples an optical microscope was used to see the differences in sample precipitation.

The microscope pictures and element analysis for the powder samples were analyzed using scanning electron microscope (SEM). The analyses were performed at Aalto University. The samples were spread on a sample holder made of chromium using two-sided tape made of conductive carbon. Before spreading, the

samples were homogenized by mixing. Light pressure was applied to ensure adhesion. Then chromium was deposited in 15 nm layers to achieve the required surface conductivity.

The element analysis was performed along with SEM using energy-dispersive X-ray spectroscopy (EDS). It is based on the backscattered electrons and characteristic x-rays from SEM. At least three points were selected for the analysis in order to determine if there were different types of particles. An element spectrum and a report were obtained. The analysis could not separate small elements like hydrogen, carbon, nitrogen and oxygen, but sulphur, phosphorus, potassium, calcium, sodium and chloride could all be detected.

Statistical analysis for the sample included t-test that was used in hypothesis testing. Correlation coefficients were calculated to factors that seemed to have linear correlations. However, there was not parallel samples or repeated experiments, and thus only several conclusions were statistically relevant.

7 Results

In this chapter the results are presented.

7.1 Enzyme 1A

Samples of enzyme 1A were dried without any additives in order to determine the effect of different cyclones and nozzle caps. The aim was to find the combination that gives largest particles. The experiment design can be found from Table 7. The samples named in this work were in the form of JR-X-Y where X represents the experiments design number (not the perform order) and Y is a running number.

Table 7. *Experimental design in order to select cyclone type and nozzle size.*

	Nozzle cap size (µm)	
Cyclone size	**140**	**150**
Small	JR-2-4	JR-2-3
Large	JR-2-2	JR-2-1

The results are divided into four sections. First section shows the results from particle size distribution analysis, second from SEM, third from elemental analysis and fourth from specific activity analysis.

7.1.1 Particle size distribution

The particle size distribution was analyzed for all the samples and size distribution curves were compared. The results can be found in Figure 6, and they suggest that the large cyclone with 150 µm nozzle cap gives the largest particles. The rest of the experiments were performed with those settings.

Another interesting result can be found in Figure 6. The scale of spray drying has an effect on particle size distribution. The same enzyme solution as was spray dried in the laboratory scale BÜCHI Mini Spray Dryer (Table 4) was spray dried in

production scale, and the resulting powders were significantly different. The mean particle sizes were 14 and 96 μm, respectively.

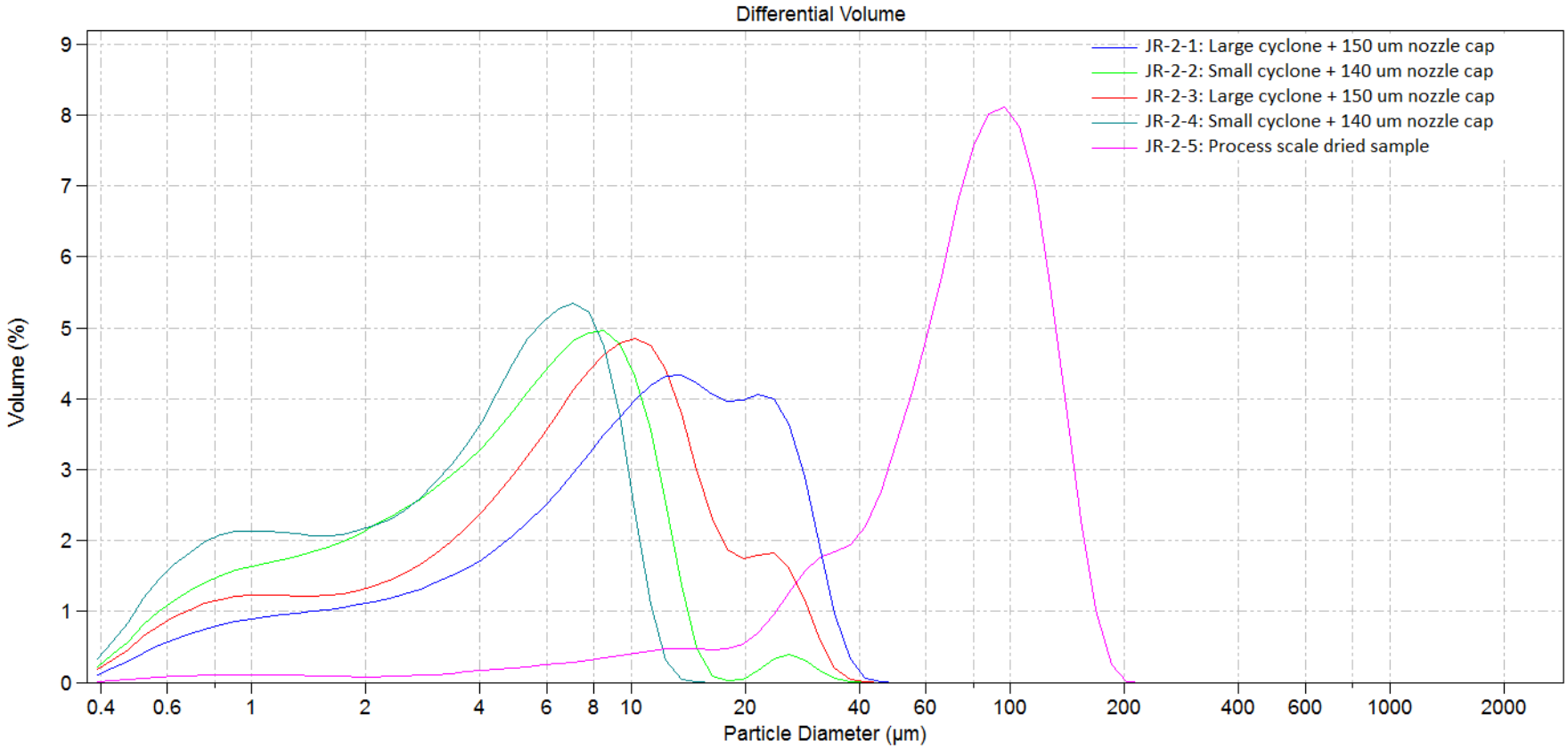

Figure 6. *Particle size distribution curves for Enzyme 1A when studying the effect of the cyclone and nozzle cap size suggest that large cyclone with 150 µm nozzle cap gives the largest particles. The sample dried in production scale differed significantly in terms of particle size from the other samples.*

7.1.2 Scanning electron microscope (SEM)

SEM pictures were taken to confirm the results gained from particle size distribution analysis. In Figure 7 the SEM pictures of samples JR-2-1 (large cyclone, 150 µm nozzle cap), JR-2-2 (large cyclone, 140 µm nozzle cap), JR-2-3 (small cyclone, 150 µm nozzle cap) and JR-2-4 (small cyclone, 140 µm nozzle cap) are shown at 1000x magnification. It is clear that the particles are generally larger in picture A) and smaller in B) and D). Therefore, also SEM pictures support the use of large cyclone and 150 µm nozzle cap, and corresponds with the particle size distribution measurements.

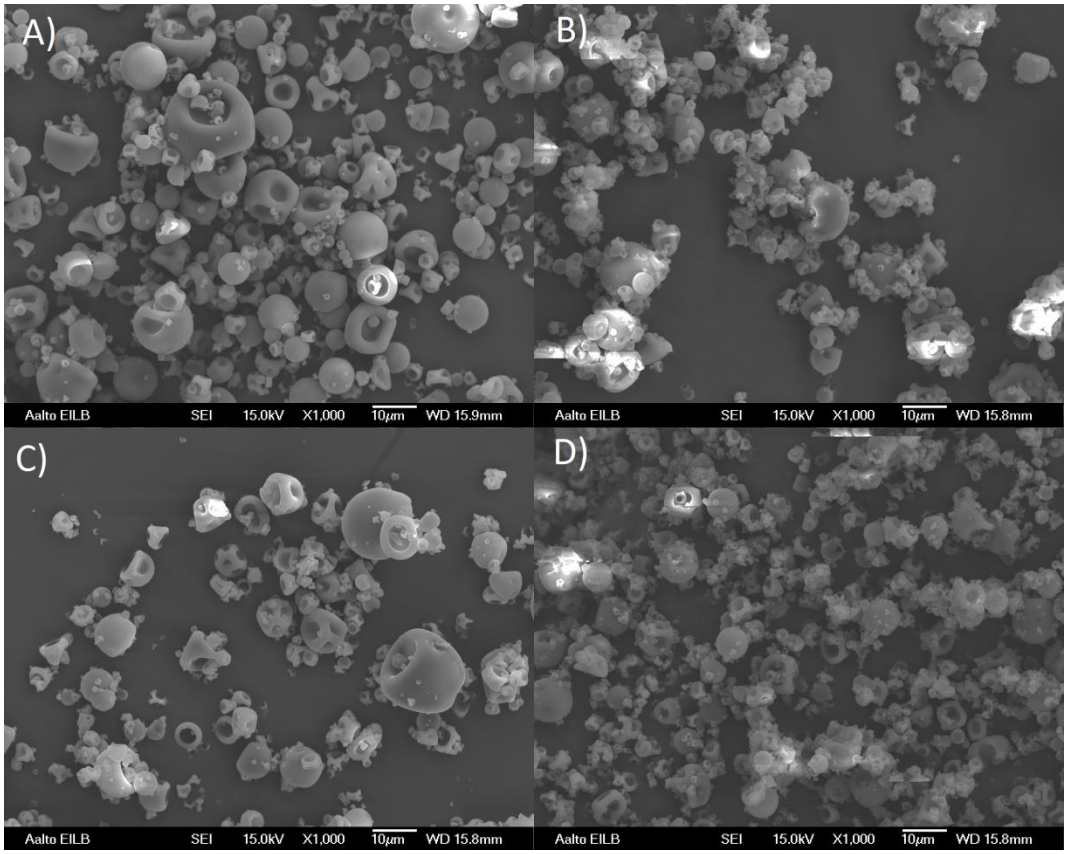

Figure 7. *SEM pictures of samples of enzyme 1A were taken at 1000x magnification in order to compare the particle sizes. As can be seen the particles in picture A) are the largest but there are several larger ones also in C) suggesting that a larger nozzle cap helps to increase the particle size. A) JR-2-1 B) JR-2-2 C) JR-2-3 D) JR-2-4.*

A SEM picture was taken of production scale dried sample of enzyme 1A to compare it with laboratory scale samples. A 1000x magnification of the particles (Figure 8) look very different compared to the particles in Figure 7. Remains of crumpled spheres can be found, but otherwise it would be hard to say that it is the same material. So, there are remarkable differences between different scales even in drying the same enzyme solutions, as was also concluded in Section 0.

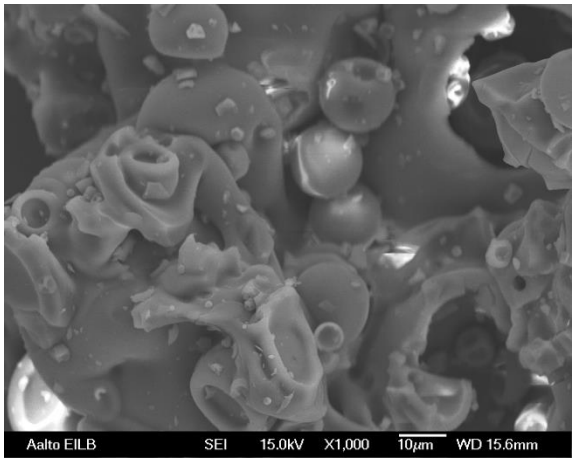

Figure 8. *The particles in SEM picture with 1000x magnification of sample Enzyme 1A from production scale spray dryer (JR-2-5) looks significantly different compared to laboratory scale and it is strongly agglomerated.*

7.1.3 Elemental analysis

Elemental analysis was performed on samples JR-2-2 (large cyclone, 140 µm nozzle cap) and JR-2-5 (dried in production scale). The analysis could only find larger molecules like sulphur, potassium and phosphorus. The relative errors in the measurements were too high to make reliable conclusions on the results. Element spectrums, data and graphs can be found from Appendix 2.

7.1.4 Specific enzyme activity yield

Specific enzyme activity yields were calculated as enzymes are produced for their activity. Even if the resulting enzyme powder had a large particle size, if the enzyme activity was lost the method cannot be considered as effective.

Enzyme activity analysis gave the specific activity yields from the drying (Table 8). There was a drop of around 10 % in the yields for all the samples, which is still at the acceptable level. The specific activity yield should be 90 % or higher.

Table 8. *Specific enzyme activity yields for the samples of enzyme 1A.*

	Nozzle cap	
Cyclone	**140**	**150**
Small	92.4 %	88.5 %
Large	88.4 %	90.1 %

7.2 Enzyme 1B

The enzyme solutions of 1B were dried with NaCl and Na_2SO_4. The concentration of the enzyme solution was varied by using filtrate, a mixture of filtrate and concentrate (ratio 1:1) or concentrate in order to see the effect of increased dry weight. NaCl concentration was 6.8 w/w % and it was blended with enzyme solutions of different concentrations to see the effect of the enzyme : additive ratio. Na_2SO_4 concentrations were 4, 5, 6 and 6.8 w/w % to compare the effect of NaCl and Na_2SO_4 and to optimize Na_2SO_4 concentration.

Samples for enzyme 1B experiments were prepared according to Table 9.

Table 9. *Experiments for enzyme 1B were done with NaCl and Na_2SO_4 as additives and using different enzyme solution and additive concentrations. An additional sample, JR-1-7, was prepared in similar fashion as JR-1-6 but with 1.5 % of methyl cellulose.*

	NaCl concentration		**Na_2SO_4 concentration**			
Enzyme solution	**0.0 %**	**6.8 %**	**6.80 %**	**6.0 %**	**5.0 %**	**4.0 %**
Filtrate	JR-1-1	JR-1-4	JR-3-1			
Mix	JR-1-2	JR-1-5	JR-3-2			
Concentrate	JR-1-3	JR-1-6	JR-3-3	JR-4-1	JR-4-2	JR-4-3

7.2.1 Particle size distribution

The particle sizes were analyzed by using mean particle sizes and D(3,2) values (details in Section 6.3.3). Most of the samples show clear trends in mean particle size but not with D(3,2) (Figure 9). D(3,2) is dependent on the surface and volume ratio, which might vary if the particles combine into large agglomerates or shrink to have large surface are but small volume.

Particle size analysis suggested that the increased protein concentration increases the mean particle size (Figure 9). The effect was greater if additives were included and the addition of Na_2SO_4 gave larger particles than NaCl. Use of methyl cellulose together with NaCl further increased the particle size. The particle size distribution curves for the samples are shown in Appendix 1.

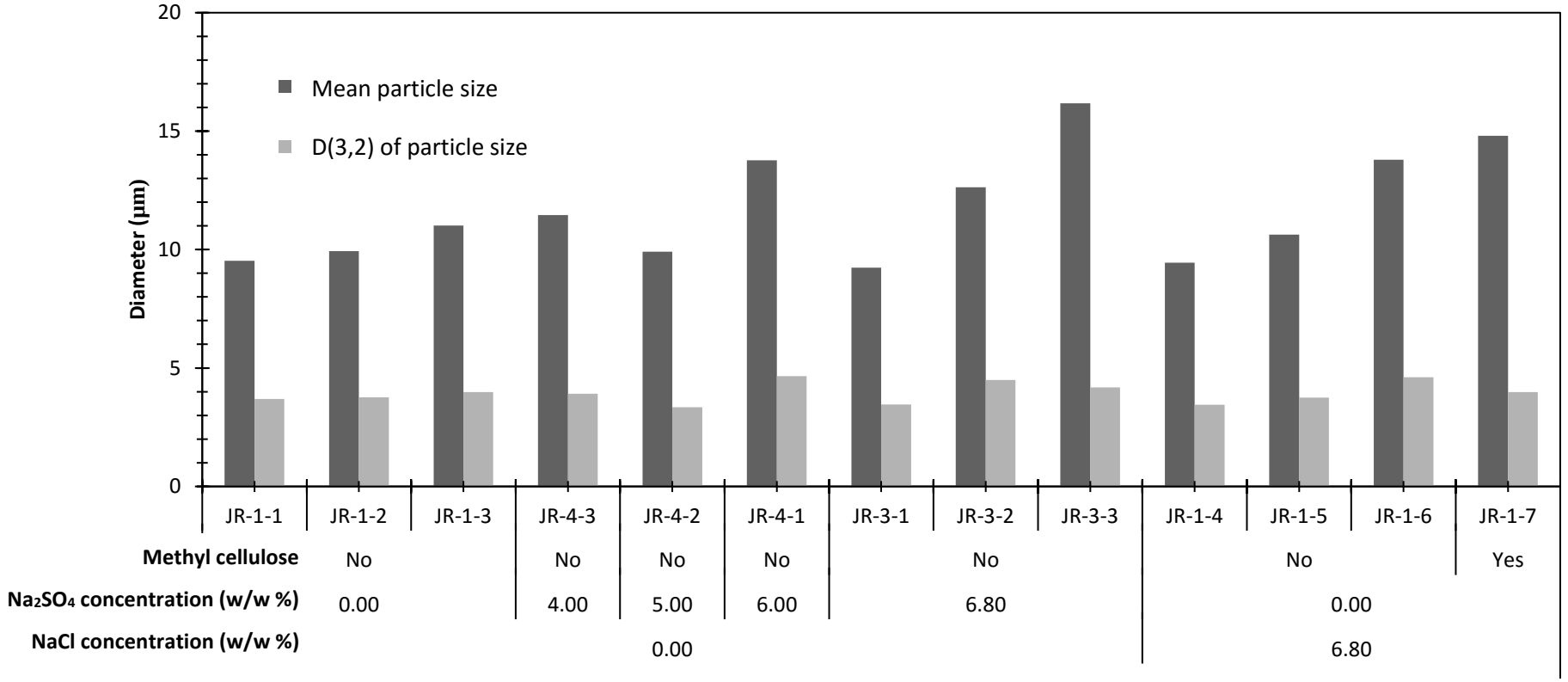

Figure 9. *The mean particle size increased when the protein concentration was raised. Without additives (JR-1-1 to JR-1-3) the effect was small compared to samples with Na_2SO_4 (JR-3-1 to JR-3-3) and NaCl (JR-1-4 to JR-1-6). Methyl cellulose (JR-1-7) further increased the particle size of JR-1-6. The sample JR-4-2 seems like it does not fit as the particle size increases from 4.0 to 6.0 and 6.8 w/w % (JR-4-1, JR-4-3 and JR-3-3, respectively).*

7.2.2 Scanning electron microscope (SEM)

SEM pictures were taken to confirm the results of particle size analysis and to see the morphology of the particles. Pictures were only taken of samples with varying enzyme concentration with or without 6.8 w/w % of NaCl added. In Figure 10, it can be seen that the number of larger particles increased when the enzyme concentration increases.

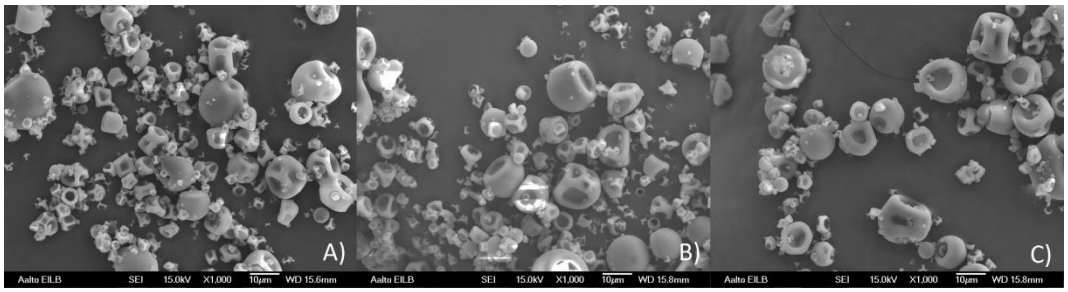

Figure 10. *SEM pictures at 1000x magnification of enzyme 1B powders showed differences in average particle size between the samples with no additives. A) JR-1-1 (Concentration factor 1) B) JR-1-2 (Conc. factor 1.5) C) JR-1-3 (Conc. factor 2)*

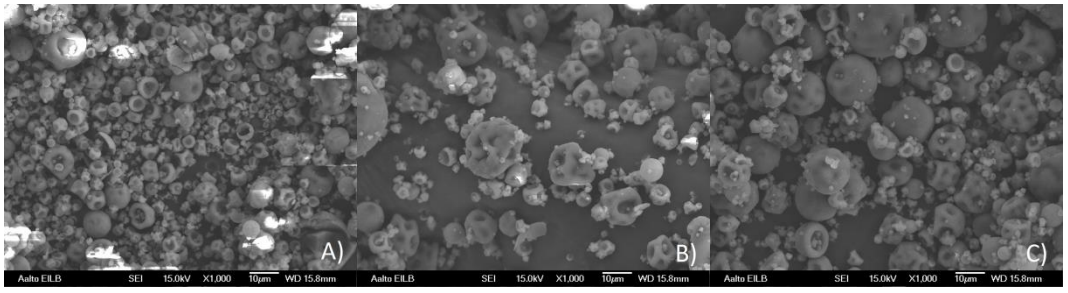

Figure 11. *A) SEM pictures at 1000x magnification reveal that increased enzyme concentration with NaCl increase the mean particle size. JR-1-4 (6.8 w/w % NaCl, Conc. factor 1) B) JR-1-5 (6.8 w/w % NaCl, Conc. factor 1.5) C) JR-1-6 (6.8 w/w % NaCl, Conc. factor 2)*

Furthermore, the morphology of the particles with NaCl is different. While the particles without NaCl look the same even though the size varies, the small particles with NaCl look different than larger particles. Also, the 2500x

magnification of the samples reveals that skin of the samples with and without NaCl are different (Figure 12).

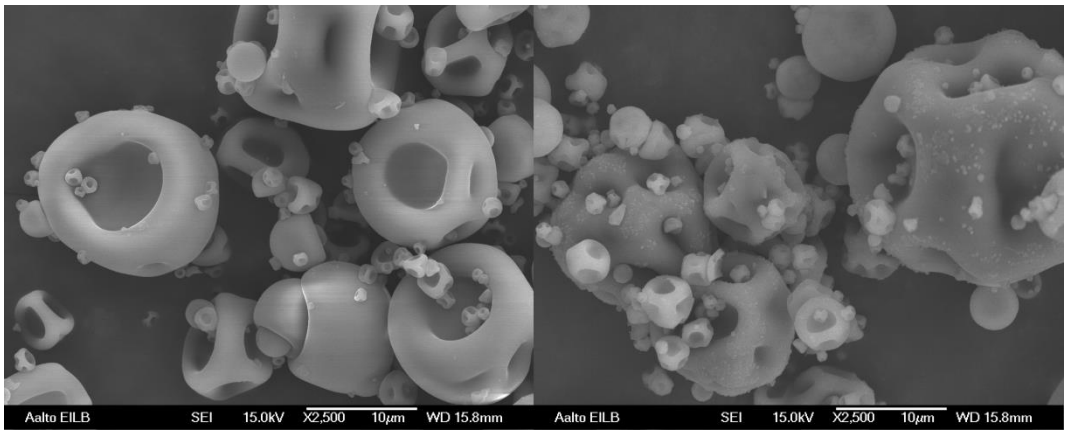

Figure 12. *SEM picture at 2500x magnification of samples JR-1-3 (left) and JR-1-6 (right) reveals the precipitated NaCl on the skin of JR-1-6.*

As was discussed in Sections 3.2 and 3.2.1, NaCl concentration on the surface of the droplet increases during the evaporation. When the concentration is high enough, NaCl precipitates and the crystals can be seen on the surface of the particles shown in Figure 12.

7.2.3 Elemental analysis

Elemental analysis was performed of all samples treated with or without NaCl. The resulting data shows if there are differences between the particle elemental contents (Appendix 2).

The samples without additives were all similar and the samples with NaCl show different ratios of sodium and chlorine. For smaller particles the ratio is closer to 1:1 than for larger particles. One explanation can be that the content of smaller particles can be analyzed throughout the particle while for larger particles the analysis might not be able to reach the center of the particles. That suggests that there is more chlorine on the surface of the particles.

7.2.4 Specific activity yield

Specific activity yield was calculated to ensure no activity was lost due to use of additives. The results of the analysis (Appendix 3) showed high errors and some results were omitted. In general it seemed like the use of NaCl increased the activity yield compared to samples without additives. Methyl cellulose did not have the same effect.

For Na_2SO_4 treated samples it was not possible to make any conclusions about activity yields. The analysis should be repeated or another analysis method considered. One reason for the problems in the analysis was that it was designed for another similar enzyme.

7.3 Enzyme 2

The experiments for enzyme 2 were designed so that the effects could be compared with the effects seen with enzymes 1B and 3. The experimental design is represented in Table 10.

Table 10. *The experiments for enzyme 2 concentrated on studying the effect of NaCl concentration and pH. Also Na_2SO_4 and methyl cellulose (MC) were tested.*

Concentration (w/w %)	**NaCl**			**Na_2SO_4**
	6.8	**12.5**	**15**	**6.8**
pH 4.8 (=no pH control)	JR-6-1	JR-6-4	JR-6-6	JR-6-3
pH 8		JR-6-5	JR-6-7	
1.5 % MC	JR-6-2			

7.3.1 Particle size distribution

Particle size of the samples was measured and the results are summarized in Table 13 showing the mean particle sizes and d(3,2) values. It can be seen that the use of Na_2SO_4 increased the particle size compared to samples treated with NaCl.

Additionally, methyl cellulose increased the particle size when used together with NaCl. When NaCl concentration is increased the effect of NaCl stayed similar but the effect of pH was stronger. For enzyme 2, the pH 4.8 seemed to be more optimal than pH 8.

The particle size distribution curves received from the analysis are represented in Appendix 1.

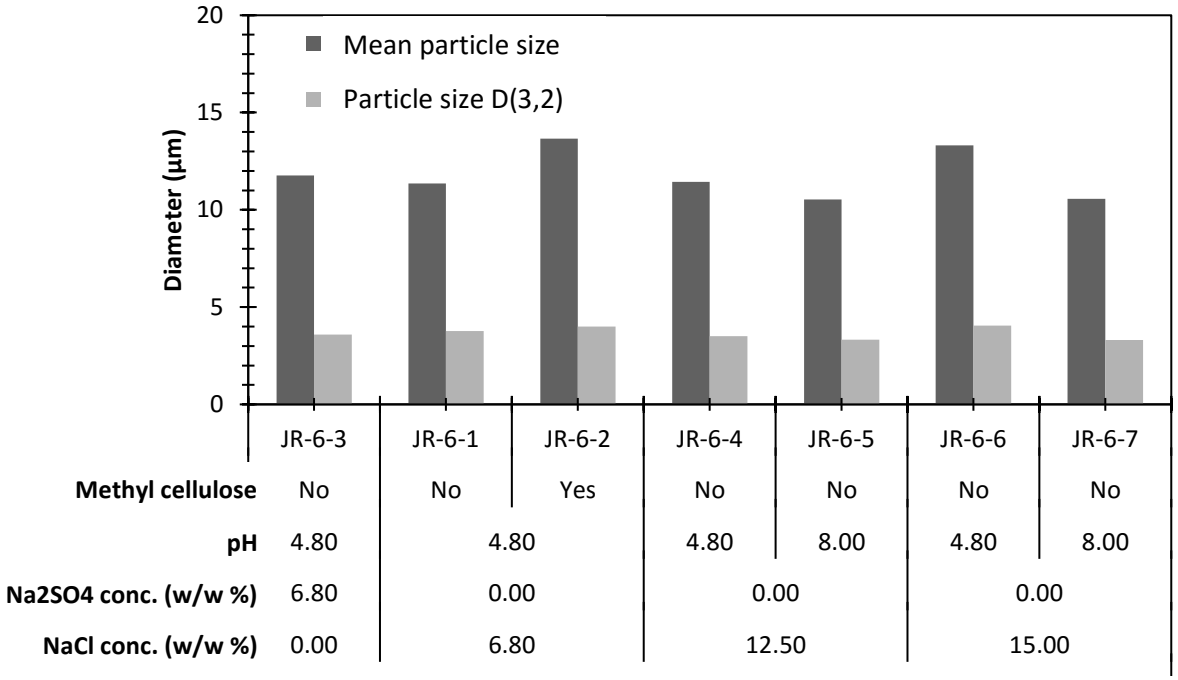

Figure 13. *The effect of Na_2SO_4 on particle size of enzyme 2 is stronger than that of NaCl. However, methyl cellulose together with NaCl increased the particle size compared to sample without methyl cellulose. Also pH has an effect as pH 4.8 seems to be more optimal than pH 8.*

7.3.2 Specific enzyme activity yield

Table 11 shows the specific activity yields calculated from the specific activity measurements. Most of the yields were over 100 % suggesting that there was some error in the measurements, even though enzyme 2 was known to be stable and high specific activity yields were expected.

Table 11. *Specific activity yields of enzyme 2 were mostly above 100 % suggesting problems in the analysis. However, it seems like the additives did not lower the activity yield of the enzyme.*

Concentration (w/w %)	**NaCl**			**Na_2SO_4**
	6.8	**12.5**	**15**	**6.8**
pH 4.8 (=no pH control)	99.6 %	103.7 %	100.7 %	-
pH 8		112.7 %	106.2 %	
MC	107.2 %			

7.4 Enzyme 3

The experiments were performed with enzyme solutions from production and two different pilot scale fermentations named as enzymes 3.1, 3.2 and 3.3. Enzymes 3.1 and 3.2 were similar production processes performed at different scales. Enzyme 3.3 was produced at pilot scale but in differing media. The enzyme produced in each of the scales was the same enzyme and produced by the same strain. The experimental designs are represented in Tables 12–14.

Table 12. *Experiments with enzyme 3.1 Sample JR-5-21 (15 % NaCl + 2 % Ca-acetate + 1.5 % methyl cellulose) is missing from the table.*

	NaCl			**NaCl + 2 % Ca-Ac**		
NaCl concentration (w/w %)	**pH 5 (=no pH control)**	**pH 8**	**pH 9**	**pH 4.5**	**pH 5,5 (=no pH control)**	**pH 8**
0	JR-5-1			JR-5-6	JR-5-10	
5	Jr-5-2			JR-5-7	JR-5-11	
7.5	JR-5-3			JR-5-8	JR-5-12	
12.5	JR-5-4	JR-5-5	JR-5-15	JR-5-9	JR-5-13	JR-5-14
15	JR-5-16	JR-5-17		JR-5-18	JR-5-19	JR-5-20

Table 13. *The experimental design for enzyme 3.2.*

	Concentrate						FIltrate
	Only NaCl		NaCl + 2 % Ca-Ac		NaCl + 2 % CaCl2		Only NaCl
NaCl concentration (w/w %)	**pH 5 (=no pH control)**	**pH 8**	**pH 6 (=no pH control)**	**pH 8**	**pH 5 (=no pH control)**	**pH 8**	**pH 5 (=no pH control)**
5	JR-5-35						
7.5	JR-5-36		JR-5-39				
12.5	JR-5-37	JR-5-38	JR-5-40	JR-5-41	JR-5-43	JR-5-44	JR-5-46
15							JR-5-47
17.5							JR-5-48
20							JR-5-49

Table 14. *The experimental design for enzyme 3.3.*

	Concentrate						**Filtrate**		
	Only NaCl			**NaCl + 2 % Ca-Ac**	**NaCl + 0.5 % ascorbic acid**		**Only NaCl**		
NaCl concentration (w/w %)	**pH 5 (=no pH control)**	**pH 8**	**pH 8.5**	**pH 8**	**pH 5 (=no pH control)**	**pH 8**	**pH 5 (=no pH control)**	**pH 8**	**pH 8.5**
5	JR-5-50	JR-5-51		JR-5-55	JR-5-57	JR-5-58			
12.5	JR-5-52	JR-5-53	JR-5-54	JR-5-56	JR-5-59	JR-5-60	JR-5-61	JR-5-62	JR-5-63
15								JR-5-64	
17.5								JR-5-65	
20								JR-5-66	

7.4.1 Particle size distribution

The largest particles of all the three groups of experiments were received from production material (enzyme 3.1). Samples with NaCl concentration of 12.5 or 15 % and no Ca-acetate gave the largest mean particle size of around 200 µm (Figure 14). The largest particles had the diameter of 1000 µm (Appendix 1, Figure 6).

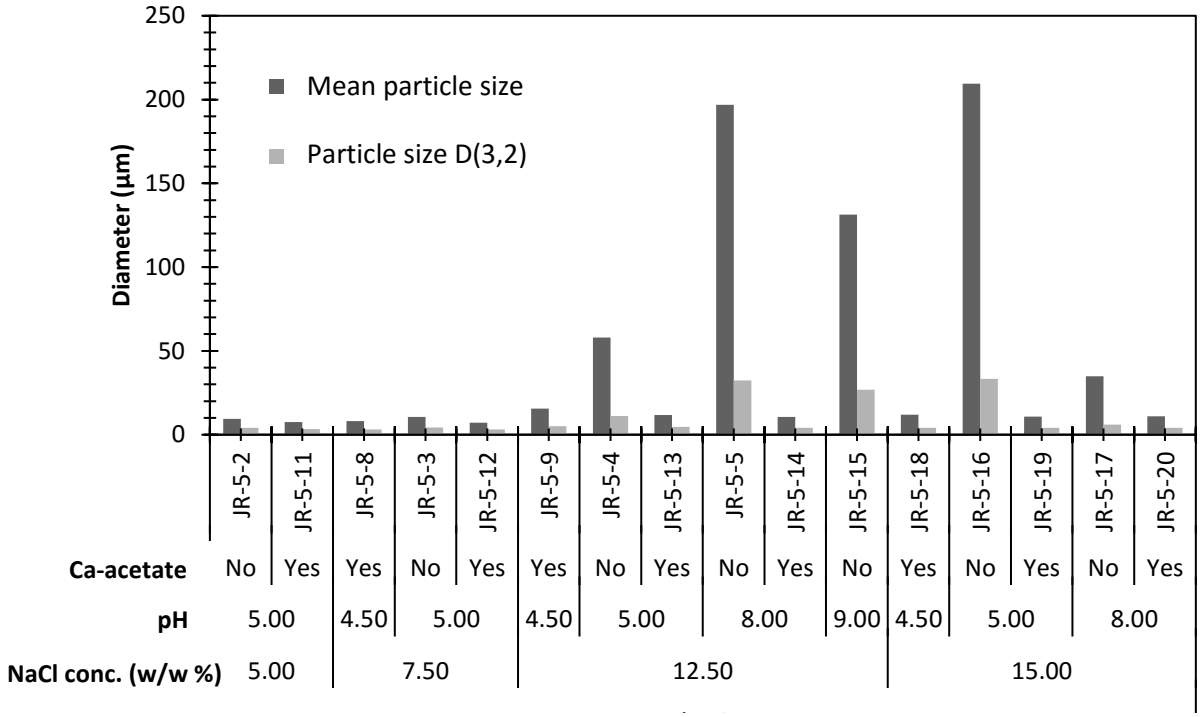

Figure 14. *Particle size was measured for all the powder samples of enzyme 3.1 and it can clearly be noticed that samples JR-5-4 (12.5 % NaCl, no pH control), JR-5-5 (12.5 % NaCl, pH 8), JR-5-15 (12.5 % NaCl, pH 9), JR-5-16 (15 % NaCl, no pH control) and JR-5-17 (15 % NaCl, pH 8) had larger particle size than other samples.*

Spray drying of the enzyme solution without additives was impossible as no powder was produced, and thus they are missing from Figure 14. Also the sample with methyl cellulose could not be spray dried.

Figure 15 reveals that the particle size of powder samples of enzyme 3.2 were over 10 times smaller than of enzyme 3.1. However, also here the NaCl concentrations 12.5 and 15 % without Ca-acetate or $CaCl_2$ gave the largest particles.

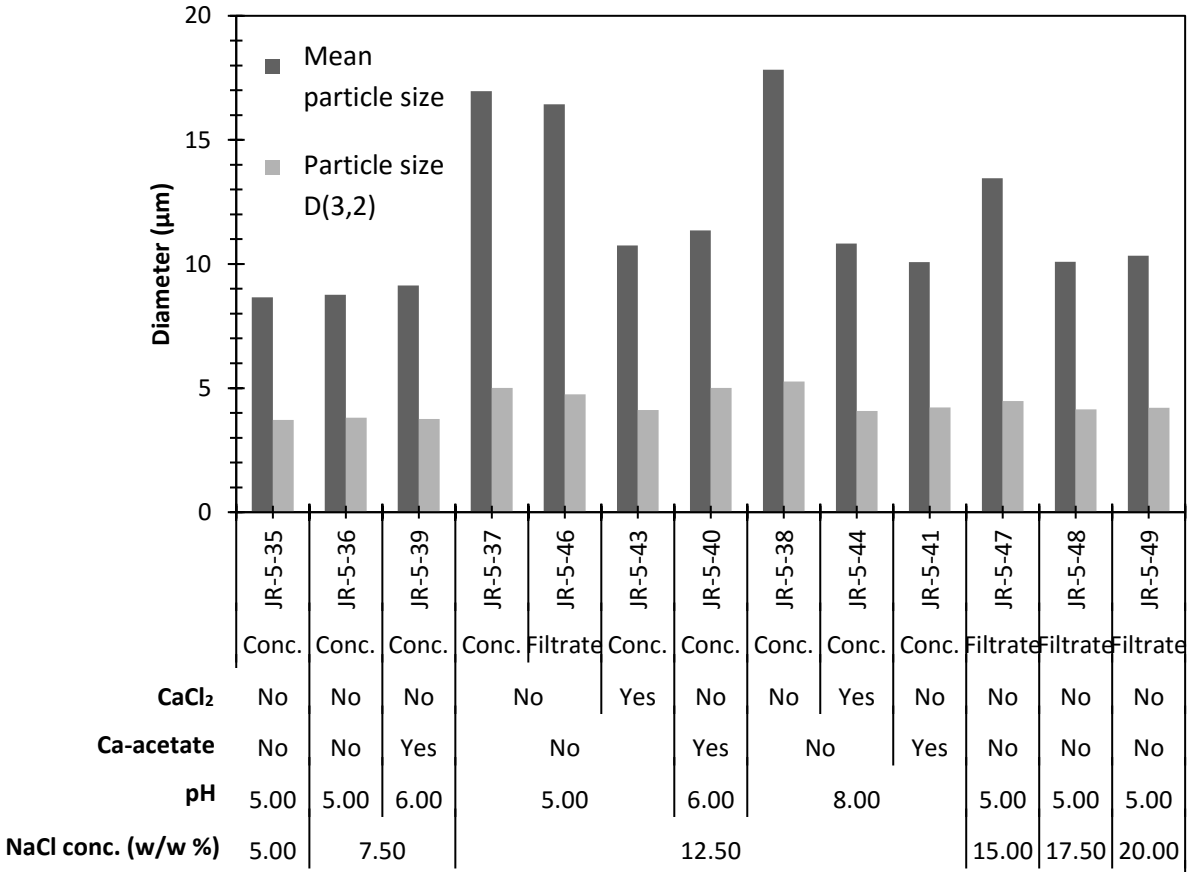

Figure 15. *The addition of 12.5 or 15 % of NaCl and not using Ca-acetate or $CaCl_2$ resulted the largest mean particle sizes for the powder of enzyme 3.2.*

Figure 15 also shows that with 12.5 % NaCl filtrate could be spray dried and large particles received, which would be beneficial in the industries where concentration of the solutions take a long time.

The mean particle size of powders of enzyme 3.3 was not strongly affected by additives (Figure 16). The use of Ca-acetate reduced the particle size as in the previous experiments. The use of ascorbic acid did not increase the particle size.

A new type of problem was discovered with the enzyme 3.3 The samples JR-5-53 (12.5 % NaCl, pH 8), JR-5-54 (12.5 % NaCl, pH 8.5) and JR-5-55 (5 % NaCl + 2 % Ca-acetate, pH 8), which had pH adjusted, turned into gels that could not be spray dried. Also other samples from concentrate had a gel-like structure, but the ones with ascorbic acid did not have that problem.

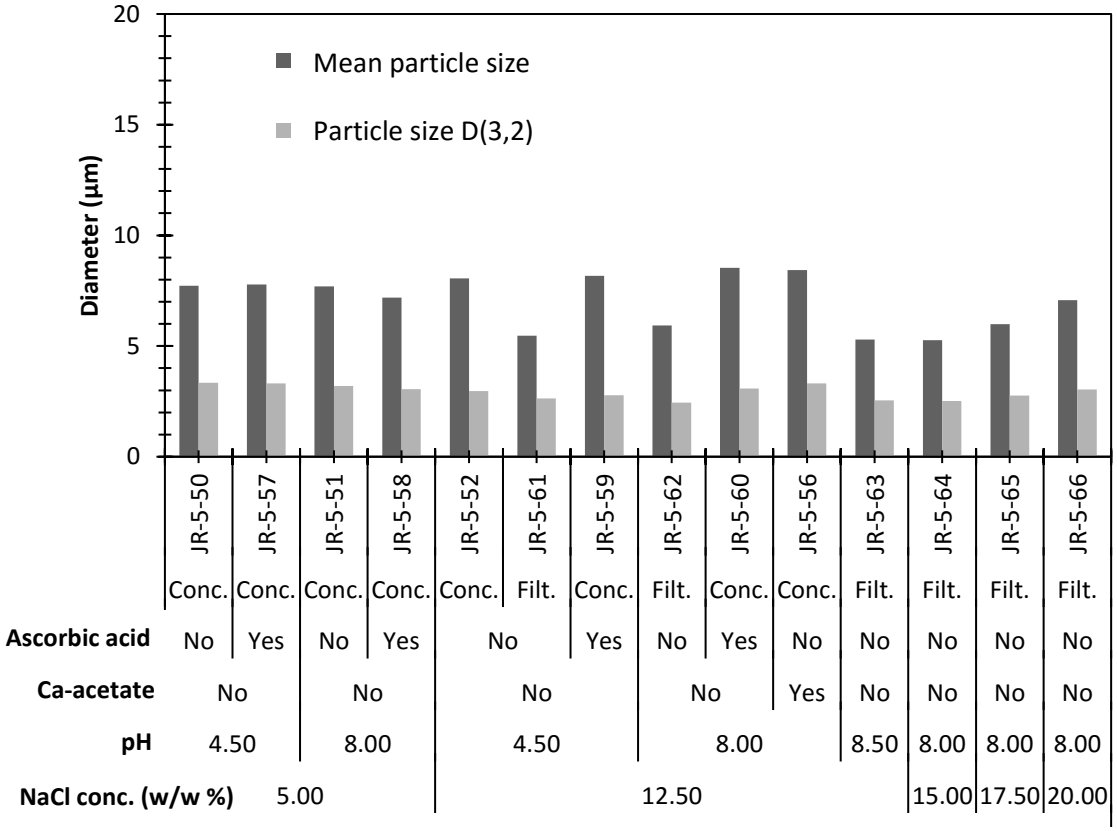

Figure 16. *The mean particle size of samples of enzyme 3.3 was not affected by additives strongly. The addition of Ca-acetate reduced the particle size as was expected. The use of ascorbic acid did not increase the particle size.*

When the results of the three experiments are combined it can be concluded that the additives can be used to change the mean particle size, but that there is an unknown factor that affects the strength of the effect.

For example, the effect of pH can be seen most clearly with enzyme 3.1. Different pH is optimal at different NaCl concentration as can be noticed from Figure 14. For

enzyme 3.1, the optimal pH for higher particle size was at pH 8–9 at 12.5 w/w % NaCl concentration while at 15 w/w % it was pH 5. Thus, we can determine that pH affects particle size, but the effect is not independent of other known factors.

Also, the same effects seen with enzyme 3.1 could not be seen with enzymes 3.2 and 3.3. The explanation could not be found, and thus more studies on the differences between these materials should be performed.

Particle size distribution curves can be found from Appendix 1. The most interesting result seen in the curves was that the samples with largest particle sizes lacked the smallest particles in the distribution curves. Those were the samples of enzyme 3.1 and using the same conditions for the enzymes 3.2 and 3.3, the same effect was not seen. One goal of this work was to find a method to remove the smallest particles. However, the factors that caused that are still unknown.

7.4.2 Scanning electron microscope (SEM)

SEM pictures were taken to confirm the results of particle size distribution analysis. The pictures of the samples with the largest mean particle size revealed that the particle size itself did not increase as much as was expected. Instead, the mean particle size was increased mostly by agglomeration, where agglomerates consisted of particles of around 10 µm in diameter.

However, the increased number of large particles also helped to increase the mean particle size. SEM pictures of samples of enzyme 3.1 with the largest mean particle size are represented in Figure 17.

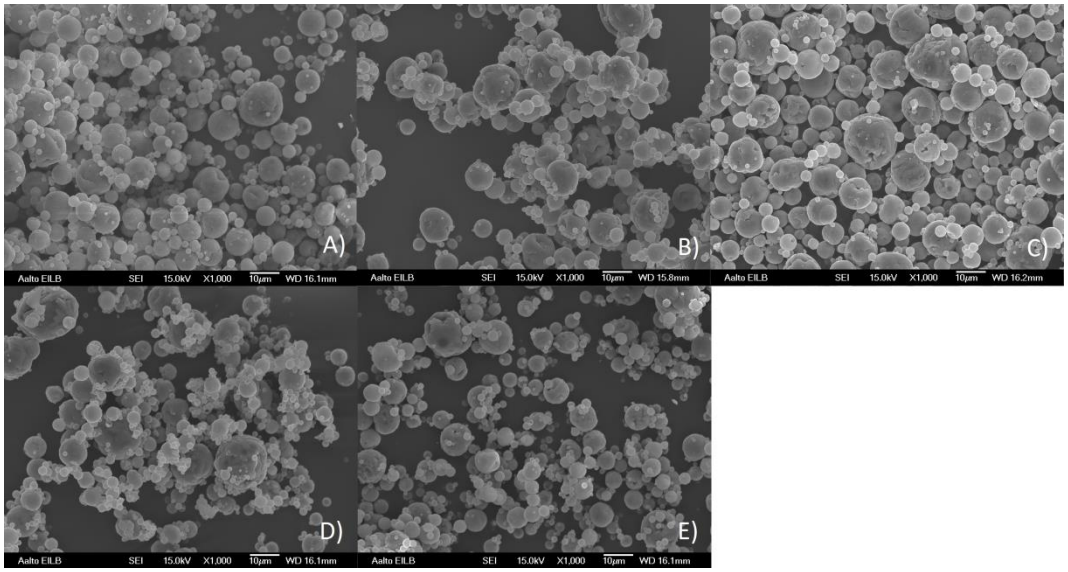

Figure 17. *SEM pictures at 1000x magnification reveal the reason why samples JR-5-5 (B) and JR-5-16 (D) give larger mean particle size from the particle size analysis. Comparing them with JR-5-4 (A), JR-5-15 (C) and JR-5-17 (E) the clear difference in agglomeration can be seen.*

Agglomeration was also a reason why several samples of enzyme 3.2 had larger particles, while samples of enzyme 3.3 did not. As can be seen in the left panel of Figure 18, smaller particles are connected to larger particles, whereas in the right panel this was not observed as strongly.

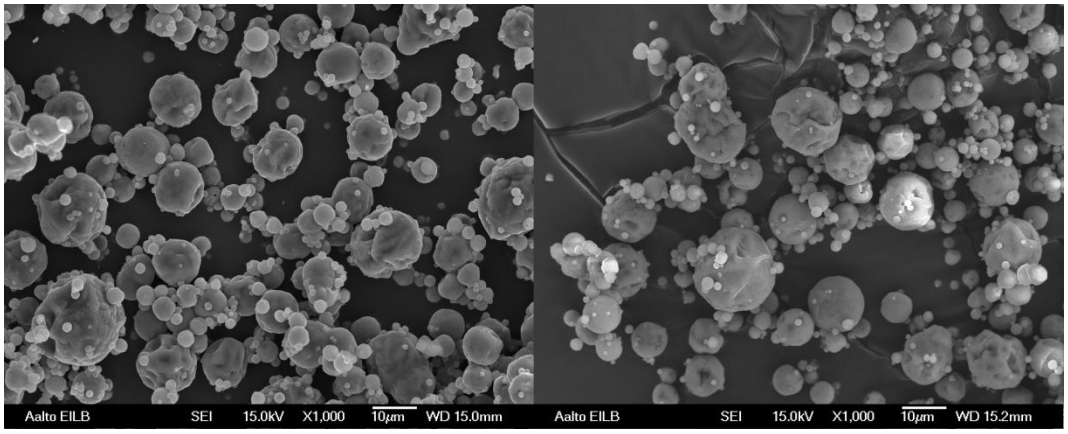

Figure 18. *SEM pictures of samples from Pilot 1 (JR-5-37, left) and Pilot 2 (JR-5-52, right) at 1000x magnification showed different amounts of sample agglomeration, even though the particles are approximately the same size. This leads to greater mean particle size in particle size analysis.*

Particles with different additive concentrations looked different. Lower NaCl concentration led to “crumbled” particles whereas higher salt concentration to smoother spheres. This seems to apply to both scales and if another additive was used along with NaCl. In Figure 19, samples of enzymes 3.1 and 3.3 with 5 and 12.5 % of NaCl were compared. Samples of enzyme 3.3 also contained 0.5 % of ascorbic acid, as the samples without it could not be dried.

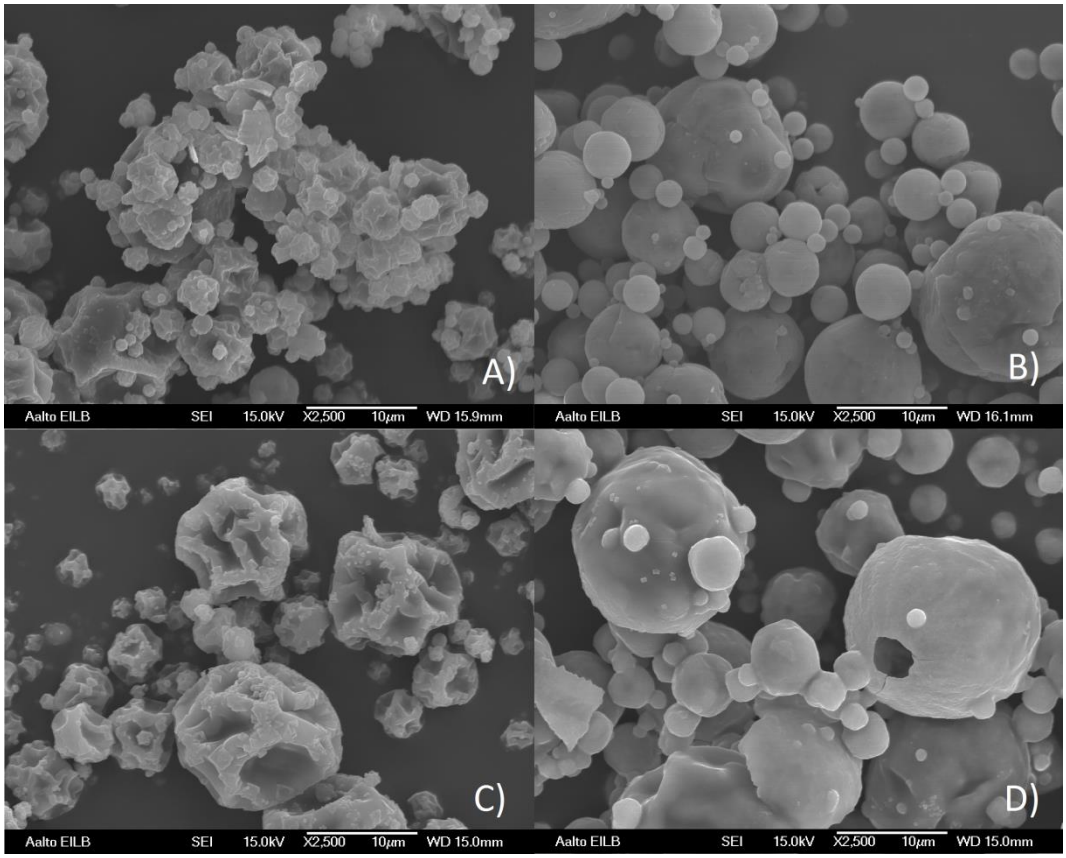

Figure 19. *SEM pictures from samples A) JR-5-2 (5 w/w % NaCl, Production 1) B) JR-5-4 (12.5 w/w %, Production 1) C) JR-5-57 (5 w/w % NaCl + 0.5 w/w % ascorbic acid, Pilot 2) and D) JR-5-59 (12.5 w/w % NaCl + 0.5 w/w % ascorbic acid, Pilot 2) were taken at magnification 2500x. They show how the lower NaCl concentration causes particles to look more "crumpled" compared to higher NaCl containing particles. Also, it can be seen in picture D) that particles seem to be hollow.*

To give a reference to particles with small mean particle sizes and highlight the importance of agglomeration, samples JR-5-5 (12.5 % NaCl, pH 8) and JR-5-14 (12.5 % NaCl + 2 % Ca-acetate, pH 8) of enzyme 3.1 are represented in Figure 20.

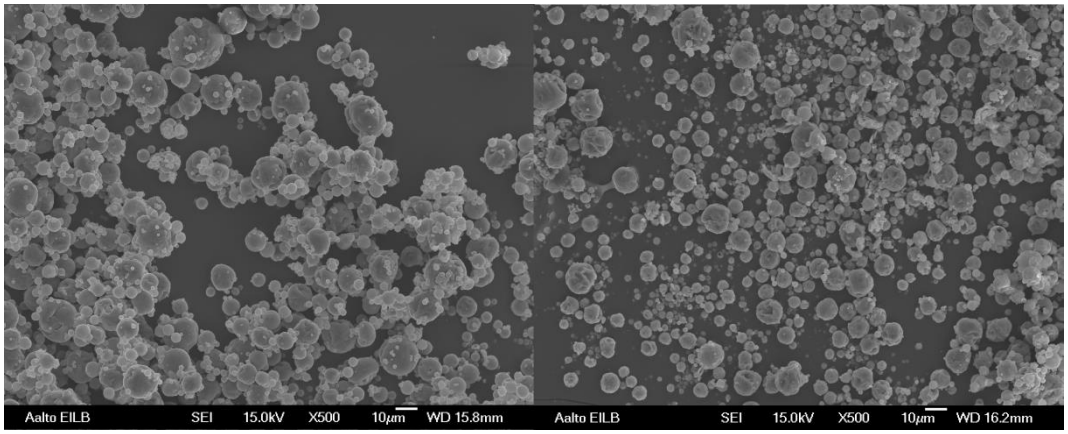

Figure 20. *2500x magnification SEM pictures of samples JR-5-5 (12.5 % NaCl, pH 8) and JR-5-14 (12.5 % NaCl + 2 % Ca-acetate, pH 8) from Production 1 show the importance of agglomeration in reduction of individual small particles.*

7.4.3 Element analysis

Elemental analysis was performed to samples several samples and the samples were chosen according to results of the particle size distribution analysis as well as due to interest in production applications. However, the received information only confirmed the presence of calcium in the samples.

The reason why the experiment was not as informative as expected was that the elemental composition was only fully analyzable for small particles. The aim of this analysis was to see the differences in particles with different morphologies but that was not possible. The elemental analysis results are represented in Appendix 2.

7.4.4 Specific enzyme activity yield

The specific enzyme activity yields were calculated for drying process and the graphs are represented in Figures 21–23. The acceptable loss was around 10 % to consider the usage of the method in possible future applications.

Samples with NaCl concentration between 5 and 12.5 % do not show significant activity losses. Ca-acetate lowers the specific activity yield of enzyme 3.1 but not of enzymes 3.2 and 3.3. Also extreme pH 9 causes specific enzyme activity loss. For enzymes 3.2 and 3.3 $CaCl_2$ significantly lowers the yield as well spray drying of filtrate samples. Therefore, the promising results gained from the increased mean particle size with filtrate samples cannot be considered as an effective method.

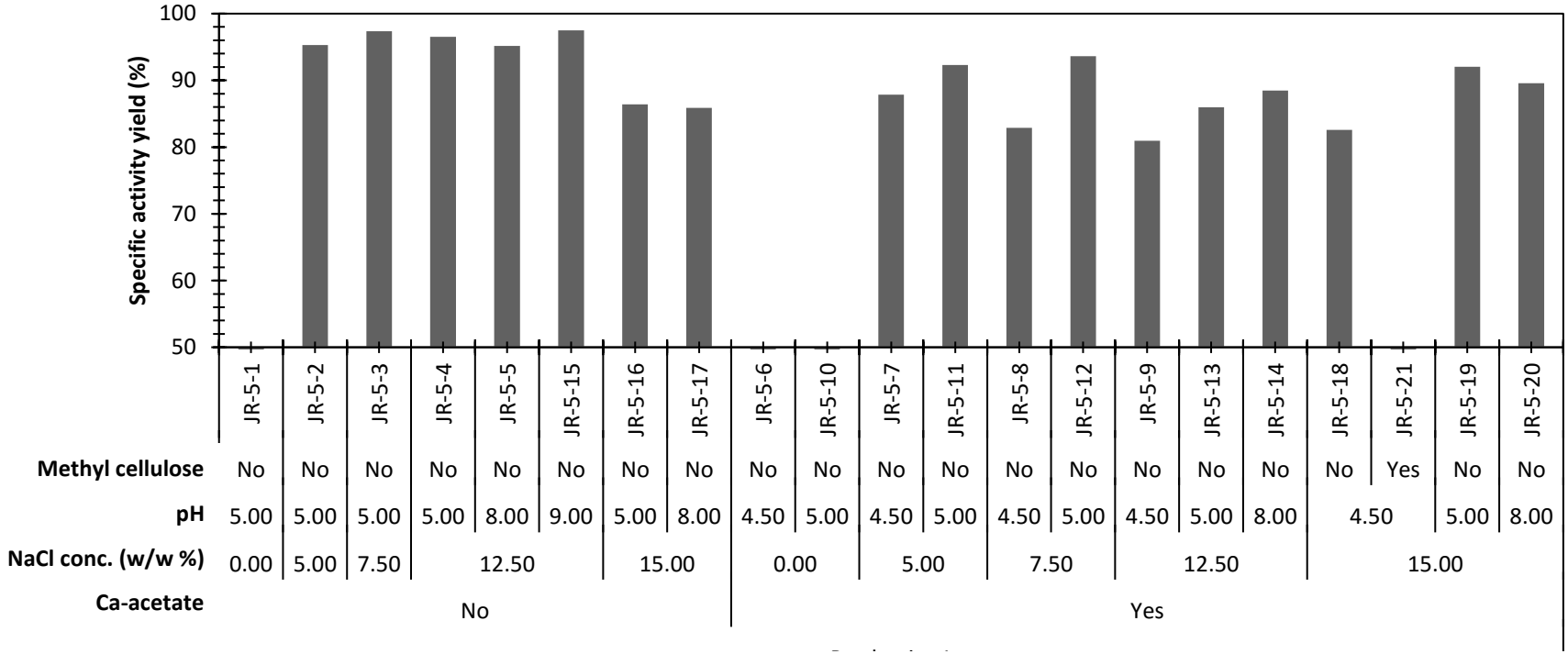

Figure 21. *Specific activity yields of enzyme 3.1 were above 90 % for samples with NaCl concentration between 5 and 12.5 % without Ca-acetate. The samples with Ca-acetate had only several samples with specific activity yields over 90 %. The samples without NaCl and with methyl cellulose could not be spray dried.*

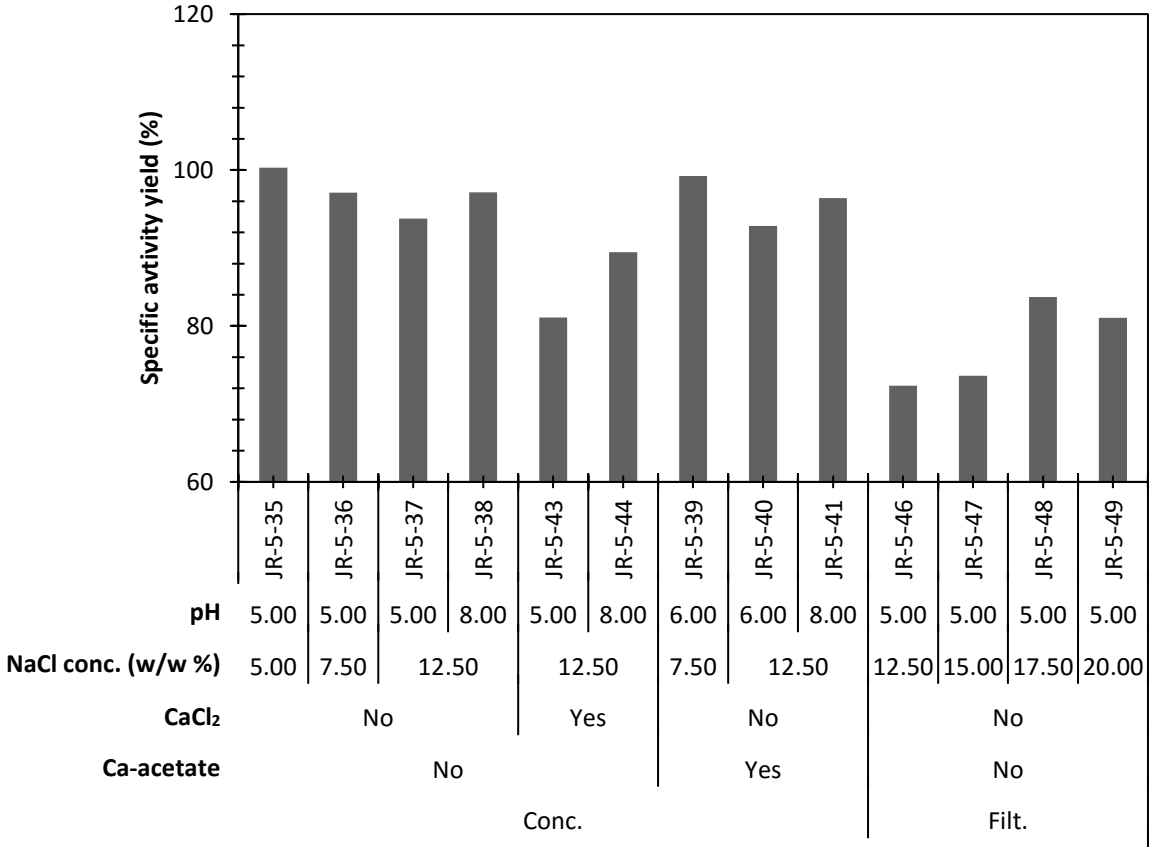

Figure 22. *Specific activity yields of enzyme 3.2 varied significantly. $CaCl_2$ lowered the yield more than Ca-acetate. All the samples with NaCl and with or without Ca-acetate had a specific activity yield over 90 %. Samples from filtrate had high activity loss.*

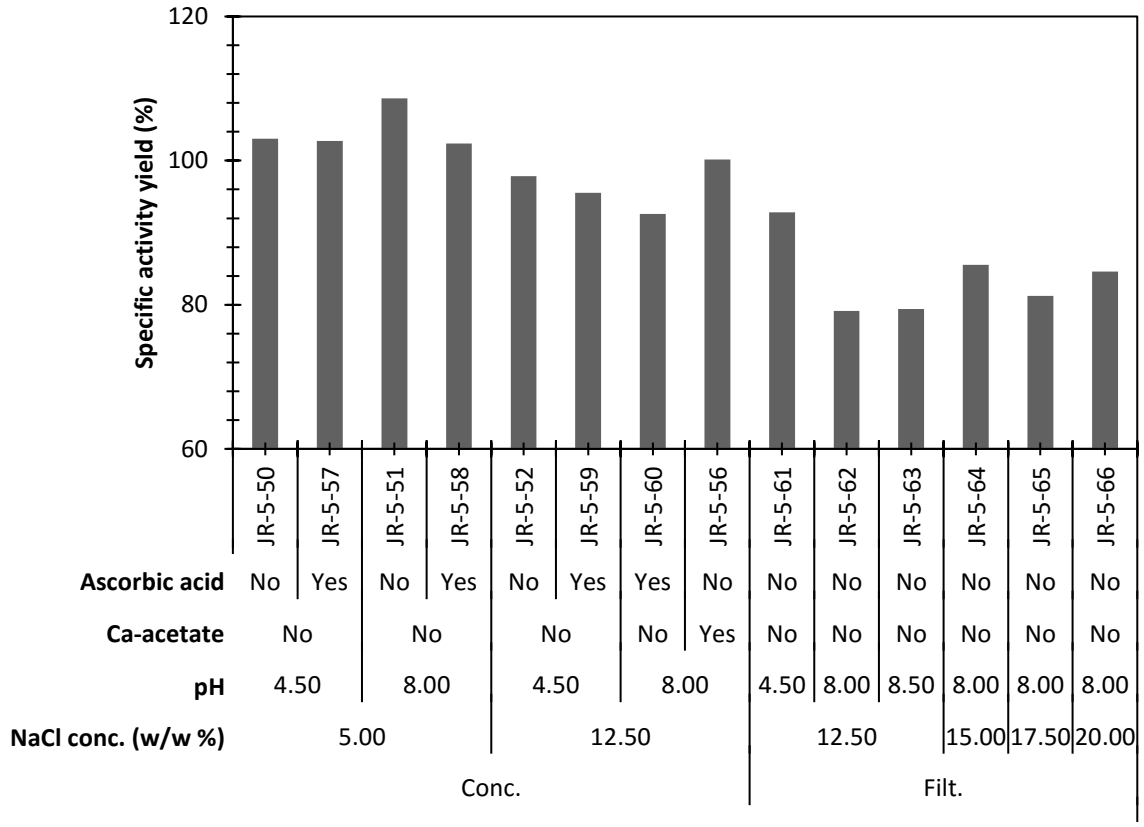

Figure 23. *Specific activities of enzyme 3.3 show that the activity yields from filtrate samples were significantly low. Also it can be noticed that the samples with ascorbic acid had slightly smaller activity yields compared to samples without.*

7.5 Material balance

Material balances were calculated for all the spray dried samples. By weighing, the feed mass and the produced powder mass could be measured. After measuring the dry weight of the feed solution and the powder, the material loss could be calculated.

The material balance can be written in the form of Equation 30.

$$ACC = IN - OUT \tag{30}$$

Figure 24 shows the chart on spray drying with BÜCHI Mini Spray Dryer B-290.

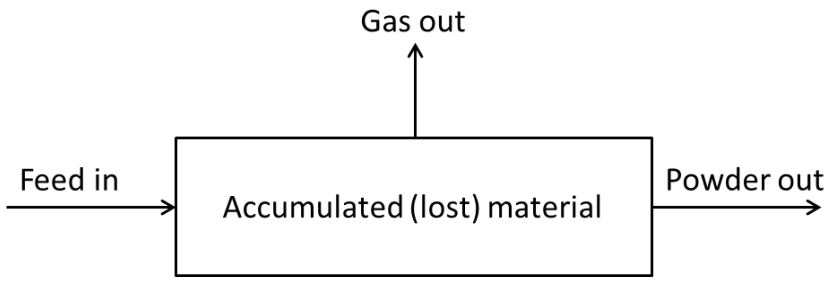

Figure 24. *Chart to describe the material balance of spray drying with BÜCHI Mini Spray Dryer B-290.*

The material loss was calculated using Equation 31.

$$Material\ loss\ (\%) = \frac{powder\ out\ (g) \times powder\ dry\ mass\ (\%)}{feed\ in\ (g) \times feed\ dry\ mass\ (\%)} \times 100\ \% \quad (31)$$

Material losses varied from sample to sample between values 5-100 %. However, the mean material loss was 32 % (median 29 %). The lowest material losses were received with the high performance cyclone and the highest losses with enzyme 3 samples without additives. Enzyme 1B had the highest mean material loss (36 %) and enzyme 1A the lowest (20 %). Enzymes 2 and 3 had mean material losses of 23 and 34 %, respectively.

The material losses obtained using BÜCHI Mini Spray Dryer B-290 do not correlate with losses of the spray dryer used in the production. The material losses in laboratory scale are generally higher, and in this study material losses were studied to see if sample treatment affected the lost materials. However, connection between material losses and mean particle sizes could not be found.

7.6 Statistical analysis

Statistical analysis was used in hypothesis testing to show the differences between samples and to prove that the results were statistically relevant. Also the correlations between parameters were studied. Statistical analysis methods were described in detail in Chapter 4.

7.6.1 Effect of calcium on particle size in Enzyme 3

Hypothesis testing was used to study the effect of calcium on particle size of Enzyme 3. The mean particle size analyzed by the particle size analyzer was used as a test parameter.

The hypothesis was tested by *t*-test for two random variables with different variances (Section 4.2).

Hypothesis was that "Ca-acetate increases the particle size", and the null hypothesis H_0 was defined as

$$\mathrm{H}_0 : \mathrm{P}_{\mathrm{no\ Ca-Ac}} = \mathrm{P}_{\mathrm{Ca-Ac}} \tag{32}$$

where P refers to mean particle size. Alternative hypotheses were

$$\mathrm{H}_1 : \mathrm{P}_{\mathrm{no\ Ca}} < \mathrm{P}_{\mathrm{Ca}} \tag{33}$$

$$\mathrm{H}_1 : \mathrm{P}_{\mathrm{no\ Ca}} > \mathrm{P}_{\mathrm{Ca}} \tag{34}$$

The filtrate samples are no included in the analysis as there were not filtrate samples including calcium. t-value was calculated to be 20.7 and $t_{0.05}$ = 1.796 (df = 11). Thus the hypothesis is rejected and the alternative hypothesis $P_{no\ Ca} < P_{Ca}$ applies. Mean, variance and the number of samples are collected to Table 15.

Table 15. *Arithmetic mean, variance and the number of samples that were used to calculate t-value for the data of specific activity yield for Enzyme 3. Samples from filtrate were not included.*

	Arithmetic mean	Variance	Number of samples
No calcium	59.2	65.4	12
Calcium	10.3	2.1	15

7.6.2 Effect of calcium acetate on specific activity yield in enzyme 3

The effect of calcium acetate on specific activity yield was studied for enzyme 3.1 data. The result can be seen from the graphs but using hypothesis testing, the statistical reliability can be ensured.

The original hypothesis was that Ca-acetate stabilizes the protein structure and therefore increases the specific activity yield. That hypothesis was tested by *t*-test for two random variables with different variances (Section 4.2).

Hypothesis was that "Ca-acetate increases the specific activity yield", but the null hypothesis H_0 was defined as

$$\mathrm{H}_0 : \mathrm{A}_{\mathrm{no\ Ca-Ac}} = \mathrm{A}_{\mathrm{Ca-Ac}} \tag{35}$$

where A refers to specific activity yield. Alternative hypotheses were

$$\mathrm{H}_1 : \mathrm{A}_{\mathrm{no\ Ca-Ac}} < \mathrm{A}_{\mathrm{Ca-Ac}} \tag{36}$$

$$\mathrm{H}_1 : \mathrm{A}_{\mathrm{no\ Ca-Ac}} > \mathrm{A}_{\mathrm{Ca-Ac}} \tag{37}$$

The arithmetic mean, variance and the number of samples of the enzyme 3.1 data are compiled in Table 16. By using Equation 13 the *t*-value was calculated for the data, and the resulting *t*-value was 2.073. Equation 16 was used to calculate the

degree of freedom (df = 11) and the significance level of 0.05 was chosen. Statistical tables gave a $t_{0.05}$-value of 1.796. As the $t > t_{0.05}$, the null hypothesis was rejected and alternative hypothesis $A_{no\ Ca\text{-}Ac} > A_{ca\text{-}Ac}$ applied. Therefore, the conclusion is that calcium acetate does not increase the specific activity yield.

Table 16. *Arithmetic mean, variance and the number of samples were used to calculate t-value for the data of Production 1 of specific activity yield for Enzyme 3.*

	Arithmetic mean	**Variance**	**Number of samples**
No calcium	91.7	31.88	7
Calcium	86.3	21.99	10

The hypothesis was tested with all the enzyme 3 samples and without including filtrate samples. The result with all the samples was that calcium acetate increases the specific yield activity. One factor affecting to that result was the low activities gained from filtrate samples and calcium acetate was not added to those. When filtrate samples were not included, the result was the same as with enzyme 3.1 samples: calcium does not increase the specific activity yield.

7.6.3 Protein concentration correlations

Values of *r* for linear correlation were calculated for enzyme 1B. The effect of increased protein concentration on mean particle size was seen in Figure 9 (samples JR-1-3 to JR-1-3) as well as the effect of Na_2SO_4 and NaCl at different protein concentrations (JR-3-1 to JR-3-3, and JR-1-4 to JR-1-6, respectively). The linear correlation lines with the r (R^2 in the figure) and the functions are presented Figure 25.

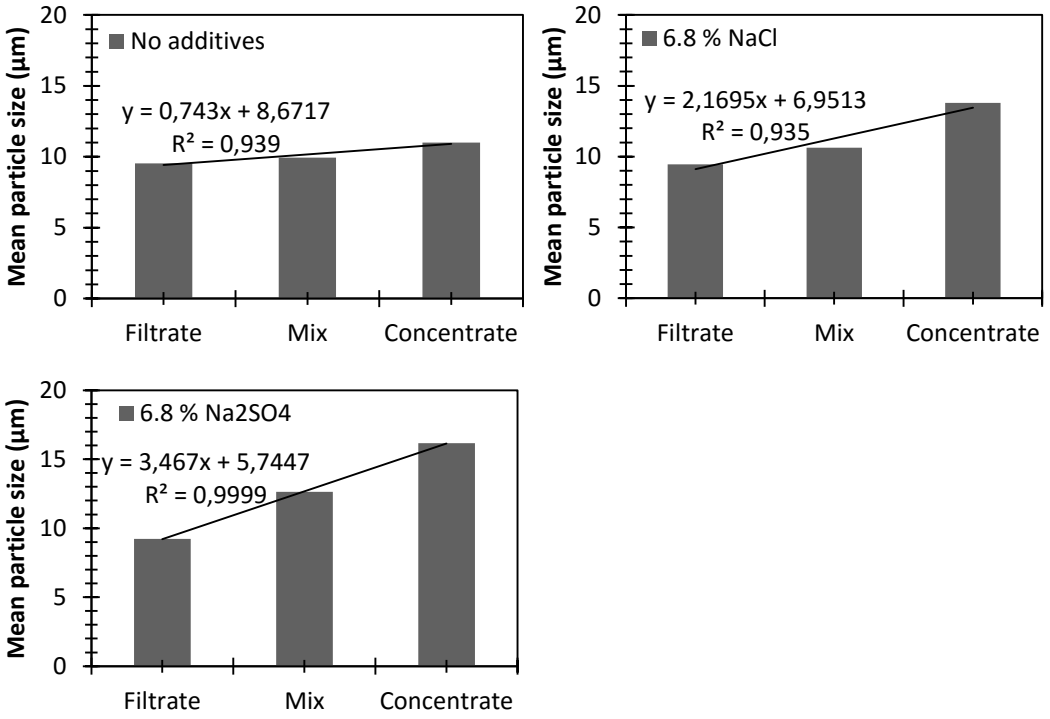

Figure 25. *Linear correlation was discovered to samples of enzyme 1B. Increased protein concentration increases the mean particle size. The effect is stronger when 6.8 w/w % of NaCl is added and further increases when using 6.8 % of Na_2SO_4.*

Linear correlations were 0.939, 0.999 and 0.935, respectively. Therefore, it is possible to say that there are linear correlations between the protein concentration and mean particle size. The reasons were described in Section 8.1.

8 Discussion

In this chapter the effect of different additives on enzyme particle size and differences of enzymes and other affecting factors are discussed and compared with the results from previous studies.

8.1 NaCl, Na_2SO_4 and pH

The use of NaCl and Na_2SO_4 as additives was one way to increase mean particle size and agglomeration. It was expected that increase in dry weight that can be

performed by adding salt increases the particle size (Table 1) and it was proven by using different enzyme solution concentrations. This applies with the previous studies[4]. However, the gelatinizing effect discovered by *Sloth et al*. was not seen in this study even at high concentrations[22].

However, another interesting phenomenon was discovered. NaCl and Na_2SO_4 precipitated proteins in the feed solution and increased the mean particle size and agglomeration. Precipitation of proteins is known to be used in spray drying to increase stability of sensitive products[20] but its effect on particle size has not been studied before.

Na_2SO_4 precipitated proteins more effectively than NaCl and caused more precipitate in the feed solution. The mean particle size of samples with Na_2SO_4 was also greater than with NaCl (Figure 9 and Figure 13). According to Hofmeister series, sulphate has higher ability to precipitate proteins than chloride[54]. Therefore it seems that the mean enzyme particle size can be increased by choosing a proper salt for enzyme precipitation.

In addition to the use of NaCl and Na_2SO_4 as additives that precipitated the enzyme from the solution, also pH caused precipitation. The effect of pH on particle size have not been studied before but it is known the effect the enzyme precipitation[54], and precipitation of enzyme was discovered to increase the mean particle size and agglomeration. Though, that should be confirmed by repeating the experiments.

As enzymes are often sensitive and degrade in the presence of high salt concentrations or extreme pH conditions, it is important to study the specific activities too. For instance, filtrate samples the solution dry weight was so low that it was necessary to add high concentrations of NaCl in order to spray dry the sample. However, that caused high losses in specific enzyme activity yields and therefore cannot be considered as an effective method.

The varying ability of enzyme to precipitate could possibly explain the differences between enzymes 1B, 2 and 3. Enzyme 3 precipitated easier than 1B while enzyme 2 hardly formed any precipitate at all. Also there were differences between the enzyme 3.1, 3.2 and 3.3 abilities to precipitate. Thus, further studies on enzyme precipitation could help to increase the particle size and confirm the connection between the particle size and ability of an enzyme to precipitate.

8.2 Methyl cellulose

The use of methyl cellulose as an additive in spray drying of enzymes was studied with enzymes 1B, 2 and 3.

In the case of enzyme 1B, high amount of powder was stuck in the bottom of the drying chamber when methyl cellulose was used (Figure 26). This suggests that the powder was too heavy for the underpressure to transfer it to cyclone. Particles can be heavy due to moisture remaining in the particles or strong agglomeration. However, strong agglomeration was not seen in SEM pictures.

In the case of enzyme 2, the use of methyl cellulose increased the mean particle size. However, the number of small particles in particle size analysis was not significantly affected.

Methyl cellulose was also added to Enzyme 3.1 with 12.5 w/w % NaCl and 2.0 w/w % of Ca-acetate. As a result the nozzle was blocked and none of the solution could be dried. That is an interesting effect as in the previous studies methyl cellulose was used to help to spray dry sticky or caking products.

Figure 26. *High amounts of enzyme powder (JR-1-7: 6.8 w/w % of NaCl + 1.5 w/w % methyl cellulose) was stuck in the bottom of the drying chamber due to stickiness and heaviness. Heavy but dry particles would have been collected into chamber sample dish.*

Methyl cellulose was expected to increase the particle size as it increases the dry weight. However, it did not increase the agglomeration as expected and therefore did not reduce the number of small particles. Also it was not expected to be sticky or cause caking and blocking as it has been used as an additive to reduce stickiness and caking[19].

8.3 Scale and fermentation differences

For enzyme 3, experiments with materials from pilot scale and production scale were done. There were significant differences in the particle sizes from the samples of the different experiments. The largest pilot scale samples had over 10 times smaller mean particle size compared to production scale samples.

The samples were prepared similarly and the effect of sample age (difference between sample preparation and drying dates) and other factors were studied to find the reason. However, it seems like there is an unknown factor to explain why the particles of enzyme 3.1 were significantly larger. It would have been interesting to repeat experiments of enzyme 3.1 with another batch from

production. However, the material was not available. Those experiments would either help to confirm the results gained in this study or increase understanding of the unknown factor or factors.

For enzyme 1A the scale difference was studied by spray drying the same material but at different scales. The product from production scale spray dryer was significantly larger and morphologically different compared to same material dried with BÜCHI Mini Spray Dryer. There were several reasons for the differences including differing nozzles and recycling of dust back to drying chamber. Therefore, it was hard to say if the results gained in laboratory scale with BÜCHI Mini Spray Dryer apply to production scale. That requires further studies.

The results and discussion above suggest that even small differences in the material can cause high variation in the end product.

8.4 Reliability

In order to draw reliable conclusions from the results, the experiments should be repeated. However, it was impossible for this work as the time and availability of materials were limited and spray drying of samples took time. To confirm the results and observations of these experiments, the number of samples should be increased significantly.

Statistically reliable results were only gained of the effects of Ca-acetate, as there were several samples with Ca-acetate that could be compared and analyzed.

9 Conclusion

In conclusion, the particle size can be affected by additives. Depending on the enzyme material the optimal additives and their concentrations vary. The most important discovery in this work was that the ability of the enzyme to precipitate

in the sample increased the mean particle size and agglomeration. As agglomeration is important in reduction of small particles, the additives that favor agglomeration helped to reduce the number of small particles in enzyme powder.

10 Further studies

Further studies are necessary in order to understand the effects of different additives better and to optimize the concentrations of additives.

Also, it seems like sodium chloride and sodium sulphate salts precipitate the proteins but some enzyme samples did not precipitate. The challenge is to find an additive that increases the agglomeration in those samples that did not precipitate.

The differences caused by different materials of the same enzyme need more research as there are still unknown factor or factors affecting the results.

Scale-up might be hard as the laboratory scale spray dryer did not model well with the production scale spray dryer. Thus, in order to apply the results on larger scale, further studies with either different laboratory scale or pilot scale spray dryers are necessary to see if the phenomena discovered in the study apply in other spray dryers.

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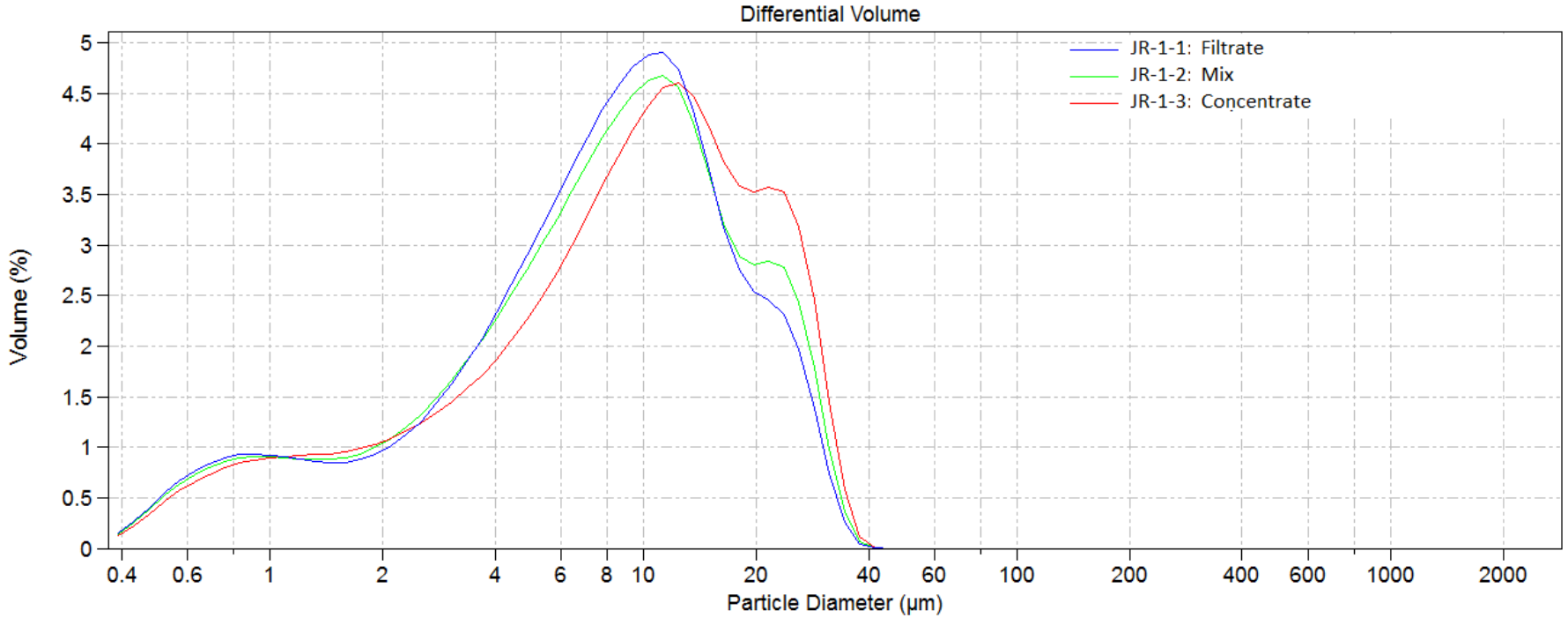


Figure 1. *The particle size distribution curve of enzyme 1B without additives did not show reduction in the number of small particles even though the mean particle size was increased when the enzyme concentration was increased. Sample JR-1-2 was a mixture of filtrate and concentrate with ratio 1:1.*

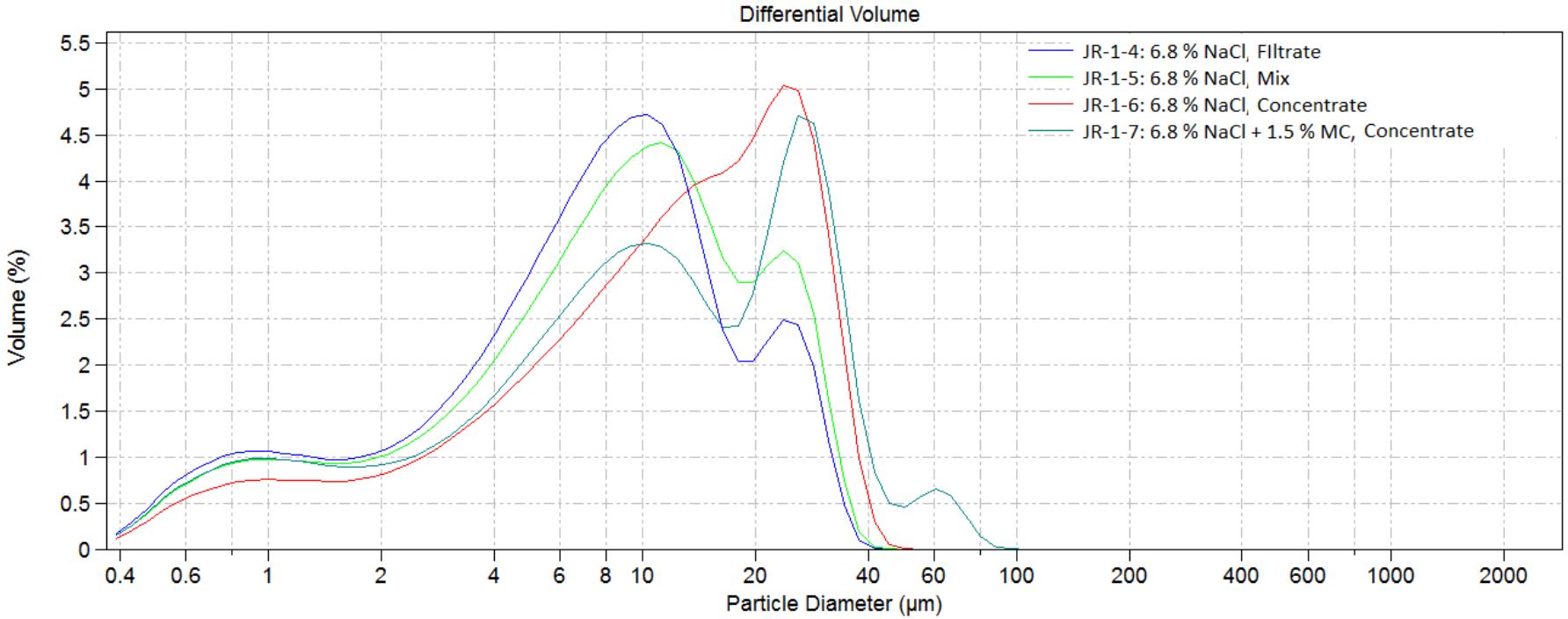


Figure 2. *6.8 w/w % of NaCl used at different enzyme 1B concentrations slightly reduced the number of small particles in the product. Methyl cellulose and NaCl together increased the mean particle size but no reduction of small particles was seen. Sample JR-1-5 was a mixture of filtrate and concentrate with ratio 1:1.*

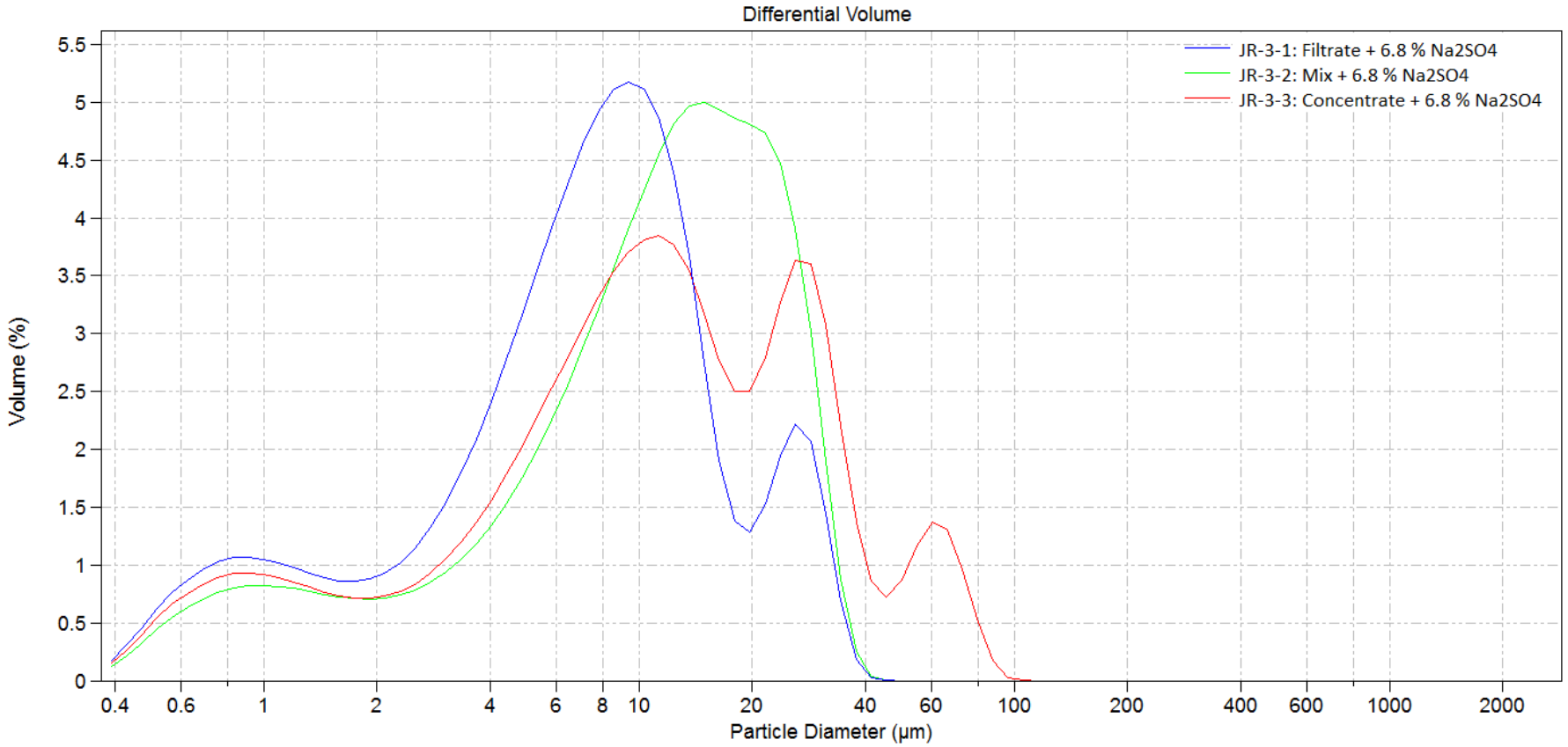


Figure 3. *The use of 6.8 w/w % of Na_2SO_4 for enzyme 1B did not significantly reduce the number of small particles at different enzyme concentrations.*

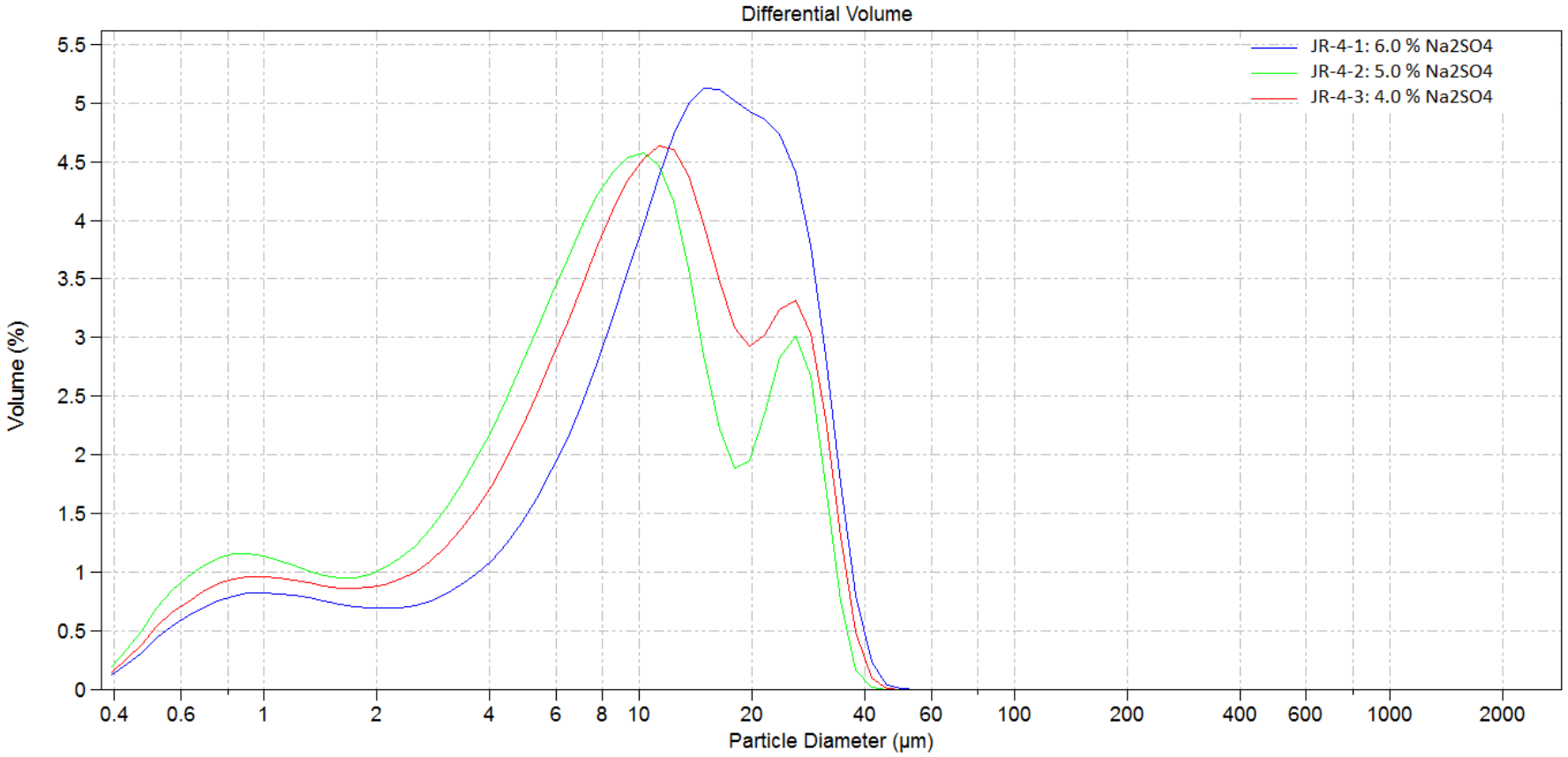


Figure 4. *Higher Na_2SO_4 concentration slightly reduced the number of small particles of enzyme 1B.*

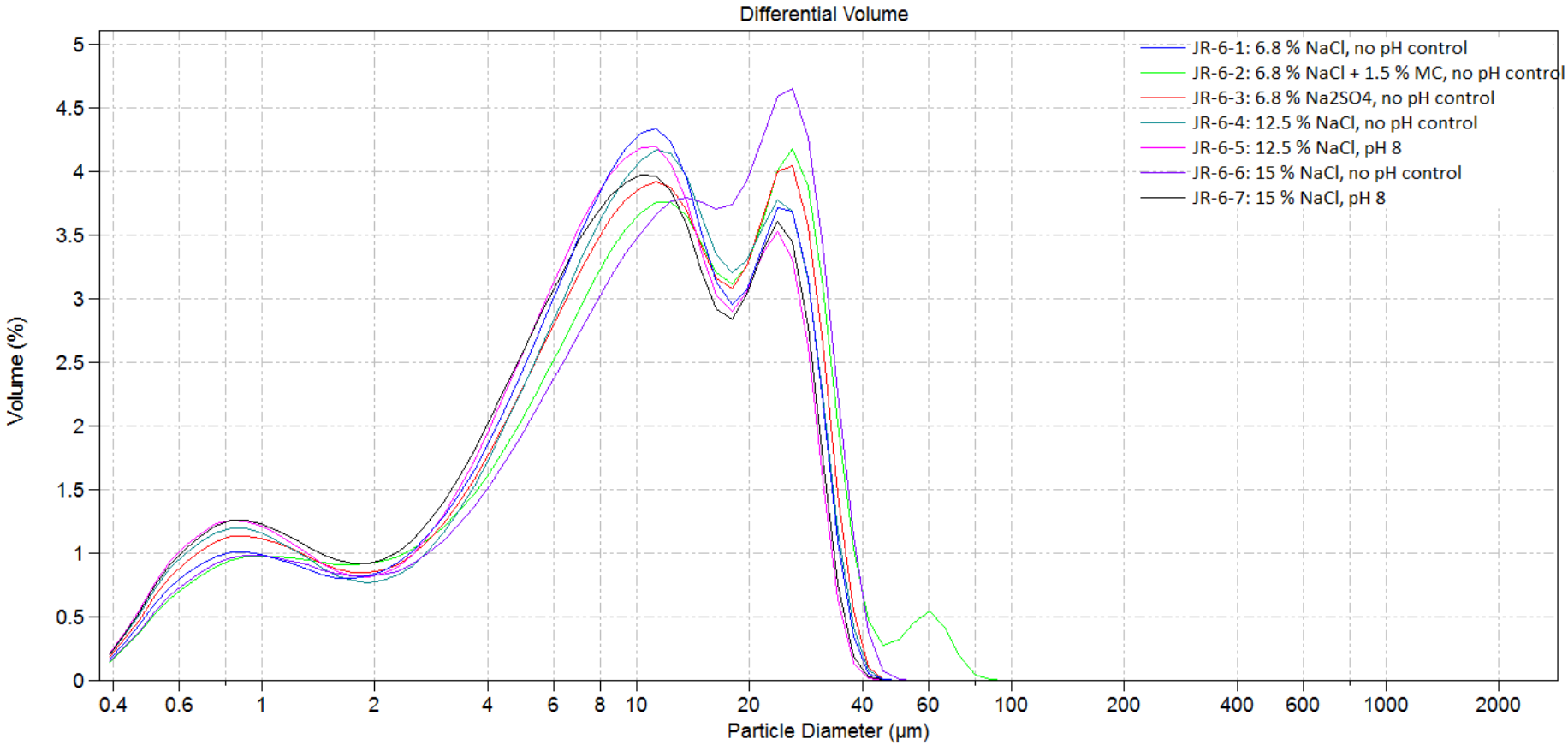


Figure 5. *The particle size distribution curves for enzyme 2 looked similar with all the additives, their concentrations and pH conditions.*

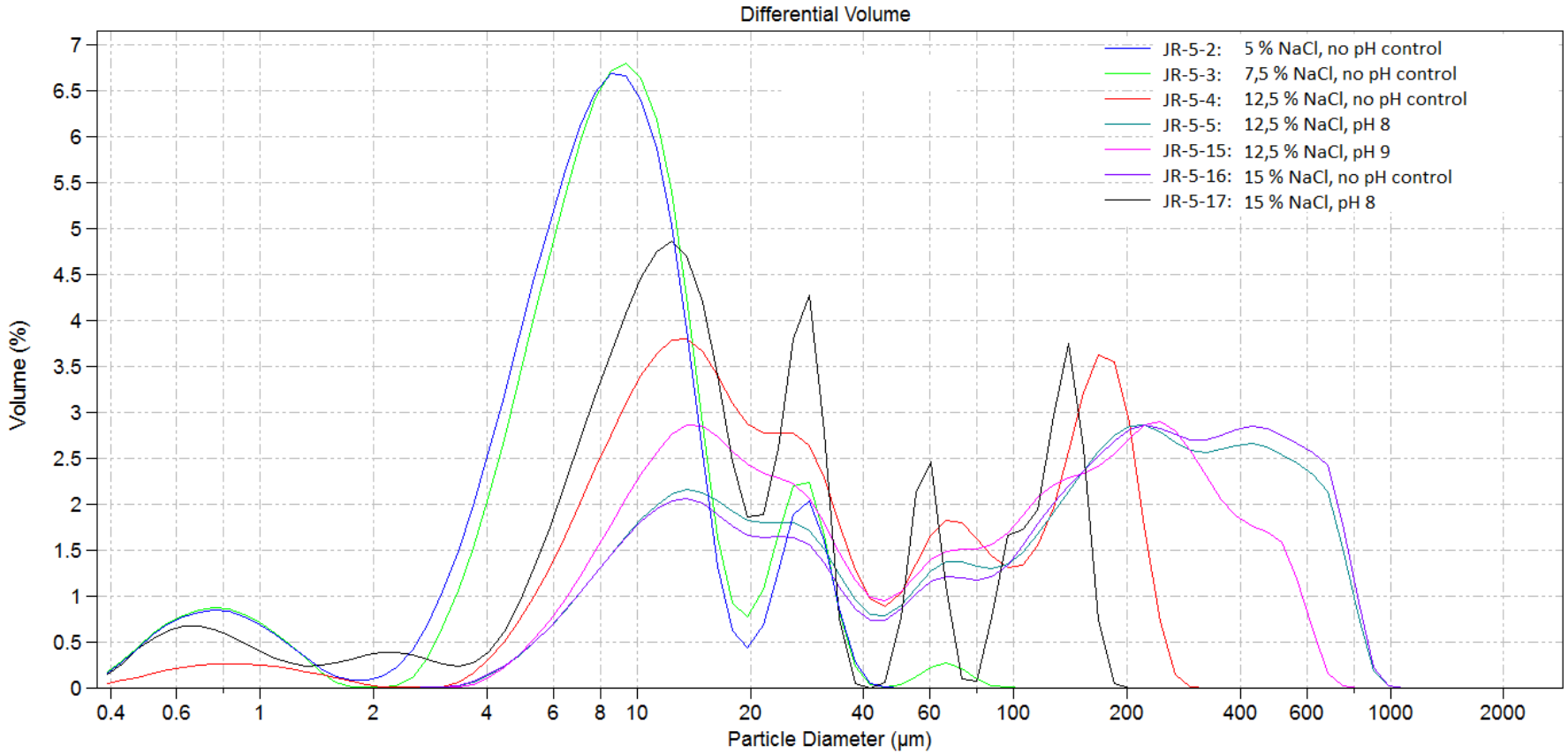


Figure 6. *The particle size distribution for enzyme 3.1 samples varied significantly at different NaCl concentrations and pH conditions. Samples JR-5-5, JR-5-15 and JR-5-16 did not have the smallest particles of less than 3 µm detected.*

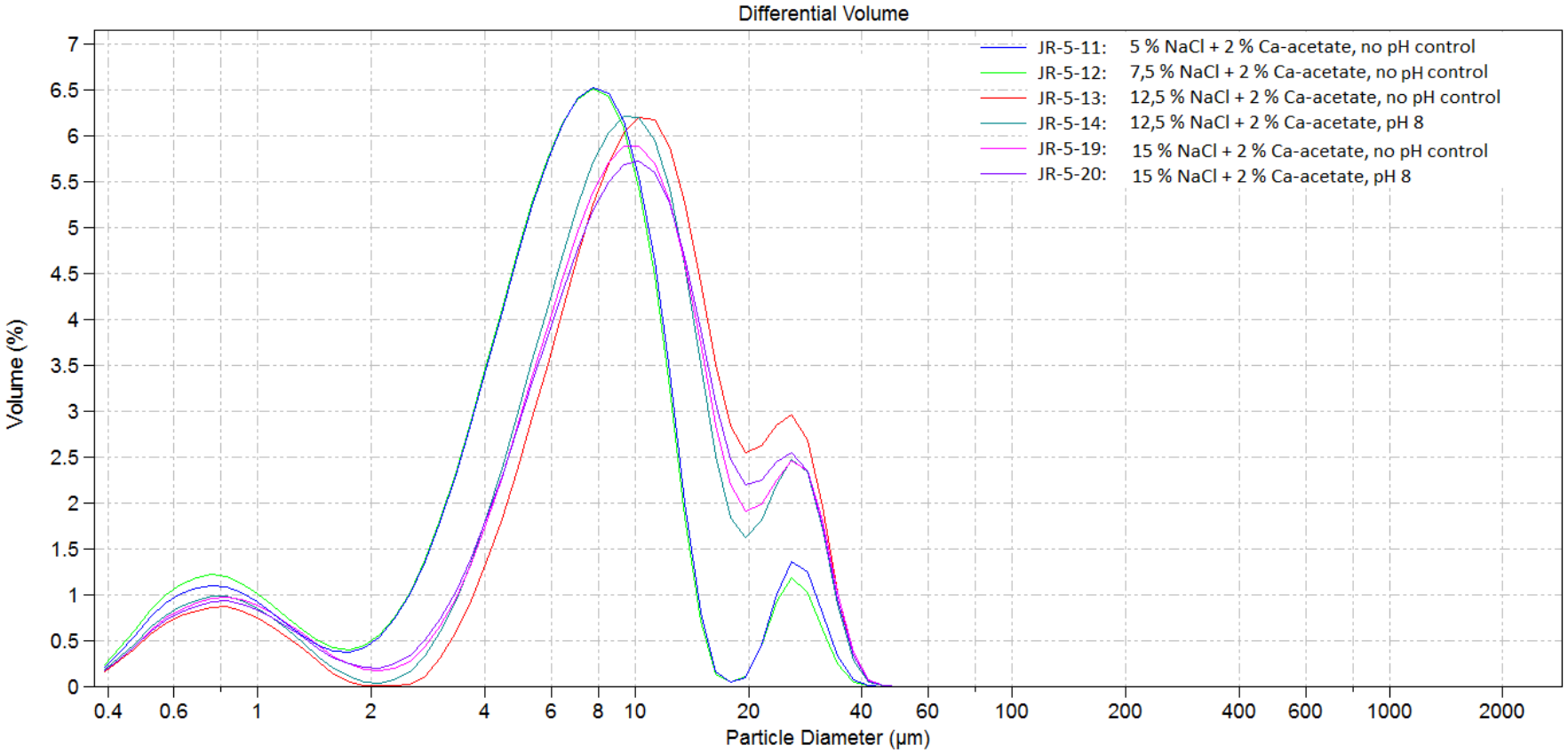


Figure 7. *The use of calcium acetate together with NaCl for enzyme 3.1 samples reduced the mean particle size compared to samples without Ca-acetate and the number of smallest particles was not reduced for any sample.*

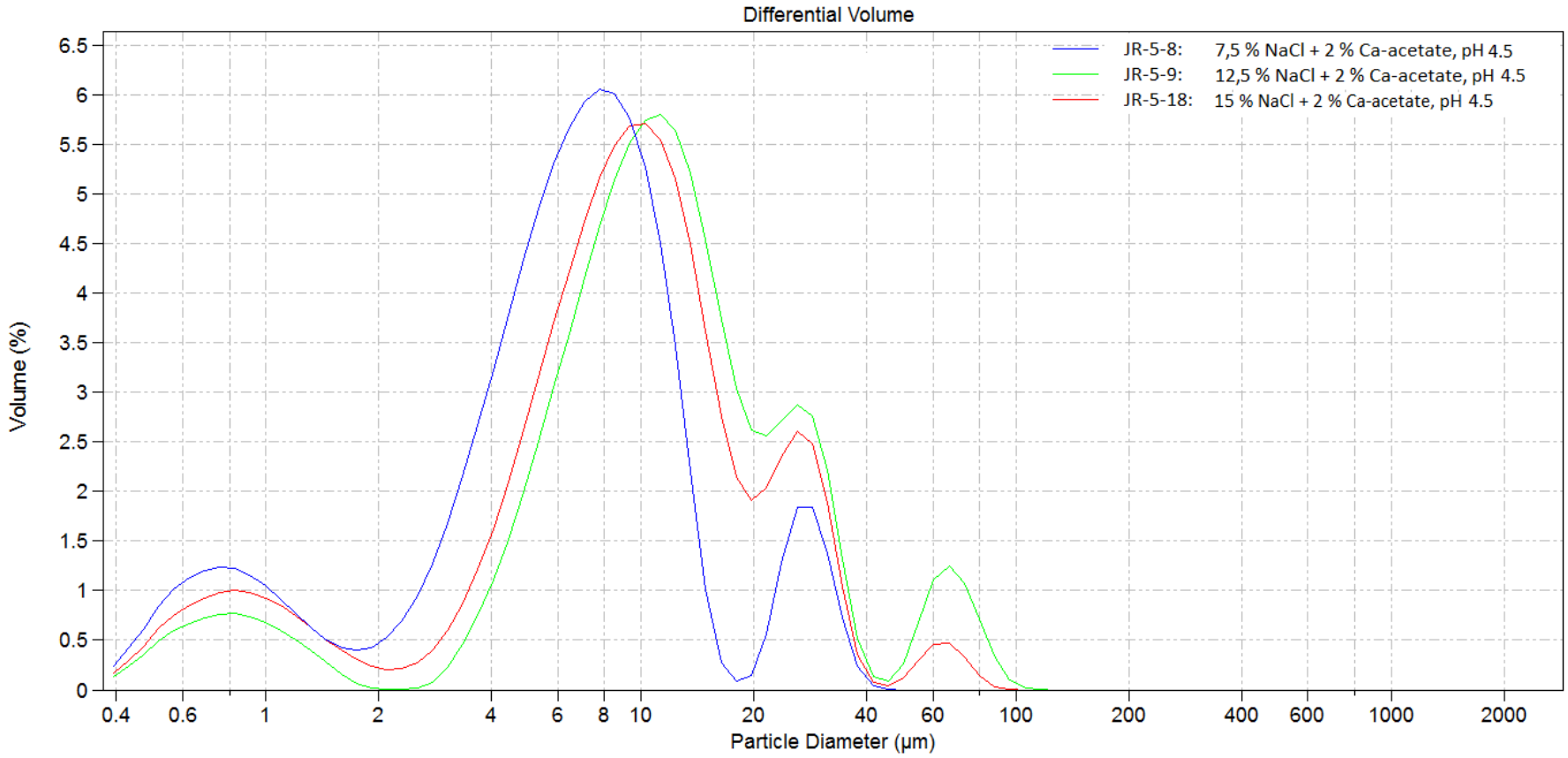


Figure 8. *The use of calcium acetate was not effective in reduction of small particles in enzyme 3.1 at pH 4.5, even though small differences can be noticed.*

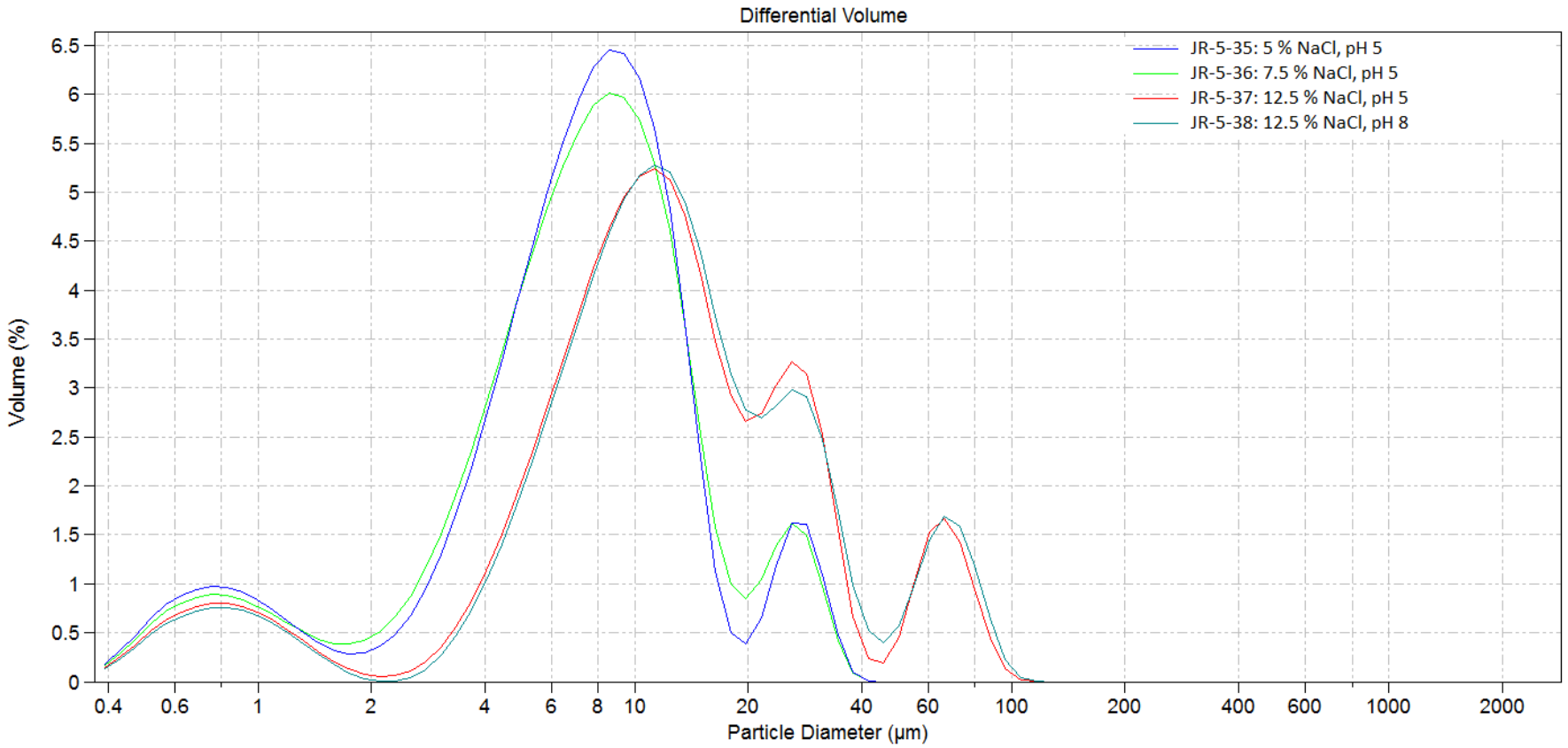


Figure 9. *The reduction of small particles in enzyme 3.2 was not effective, even though the same NaCl concentrations and pH conditions were used as with enzyme 3.1.*

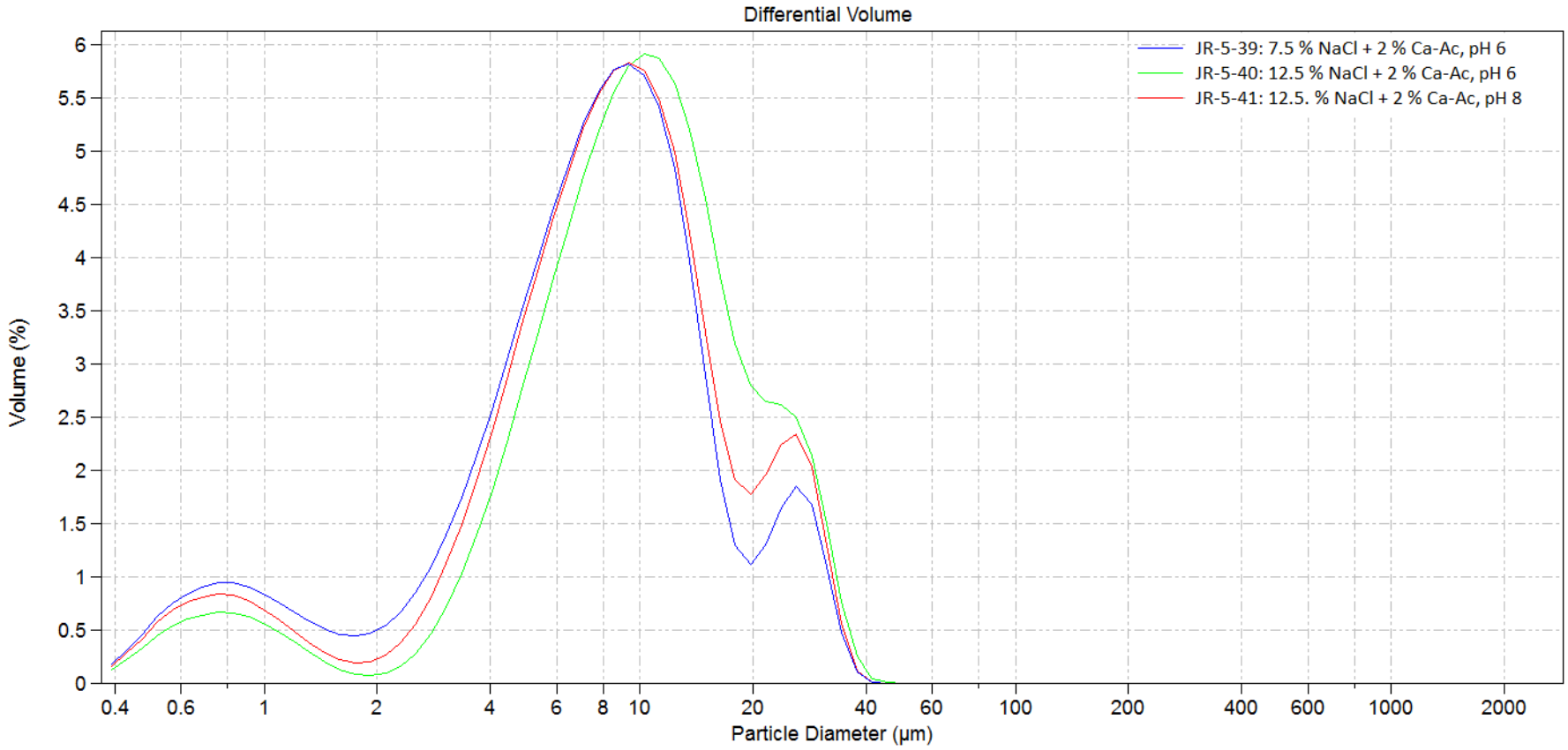


Figure 10. *The particle size distribution curves for enzyme 3.2 samples looked similar at all NaCl concentrations when used together with calcium acetate.*

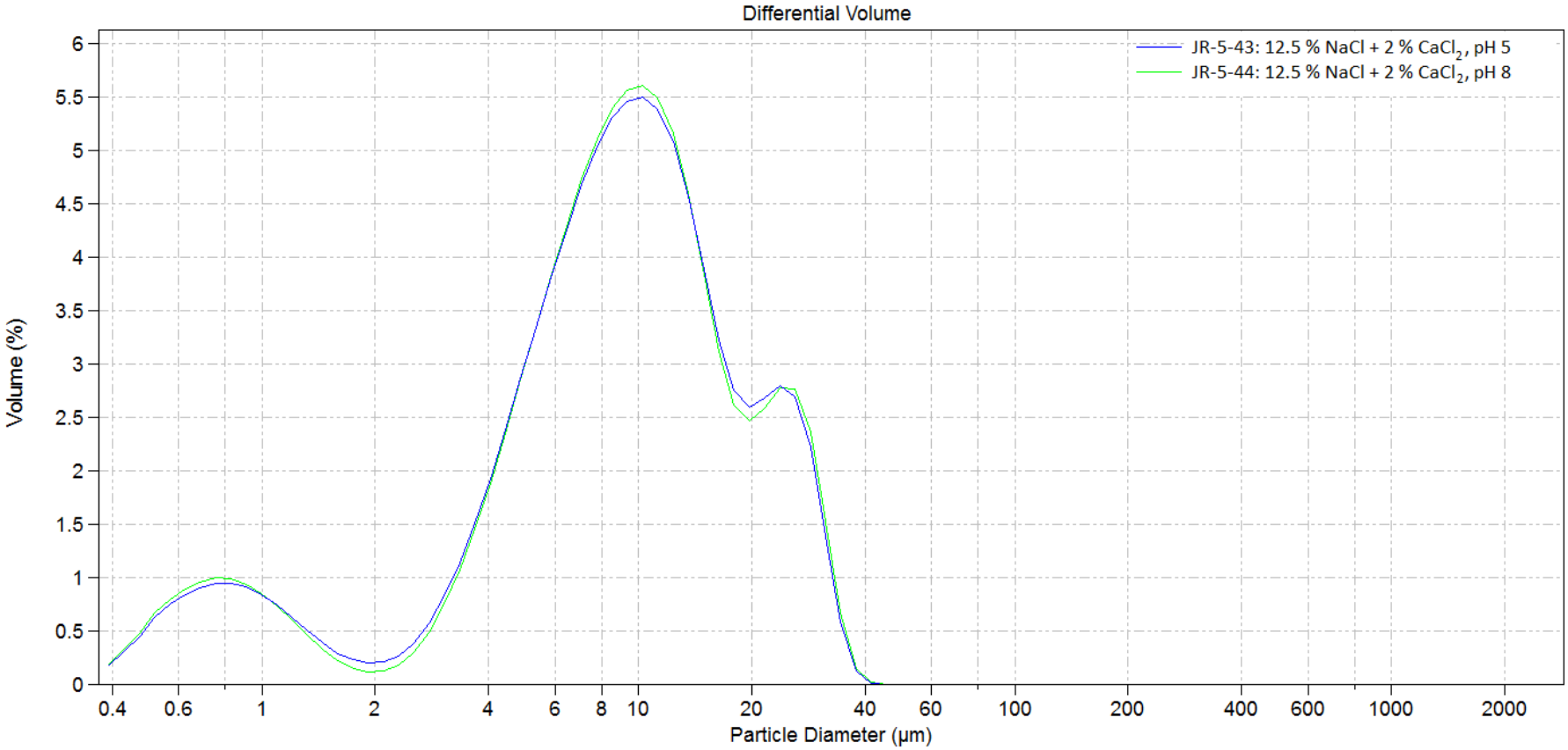


Figure 11. *$CaCl_2$ was not effective in reduction of small particles or increasing the mean particle size of enzyme 3.2 at either pH 5 or 8.*

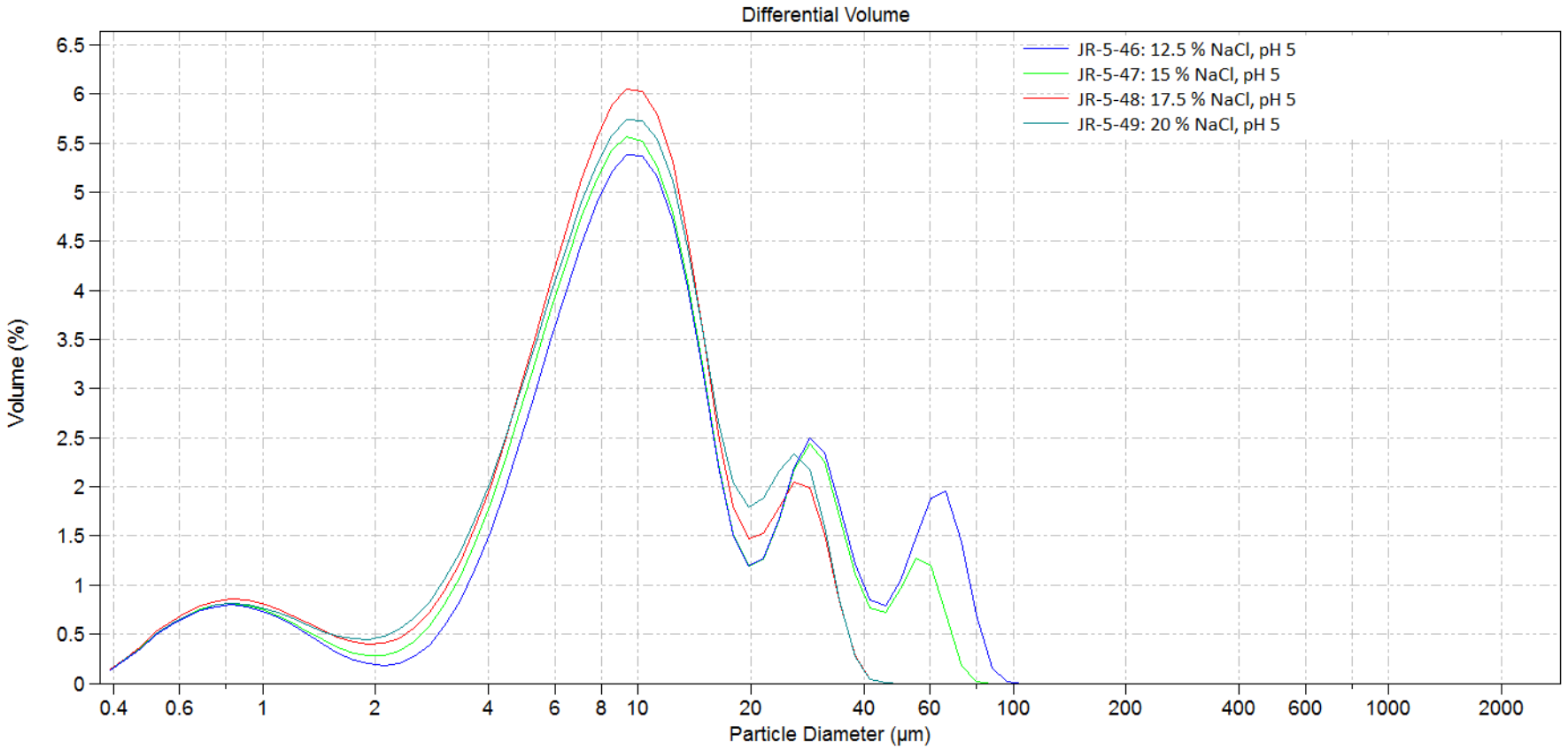


Figure 12. *The mean particle size of enzyme 3.2 filtrate was higher with 12.5 and 15 w/w % of NaCl but the number of small particles was constant.*

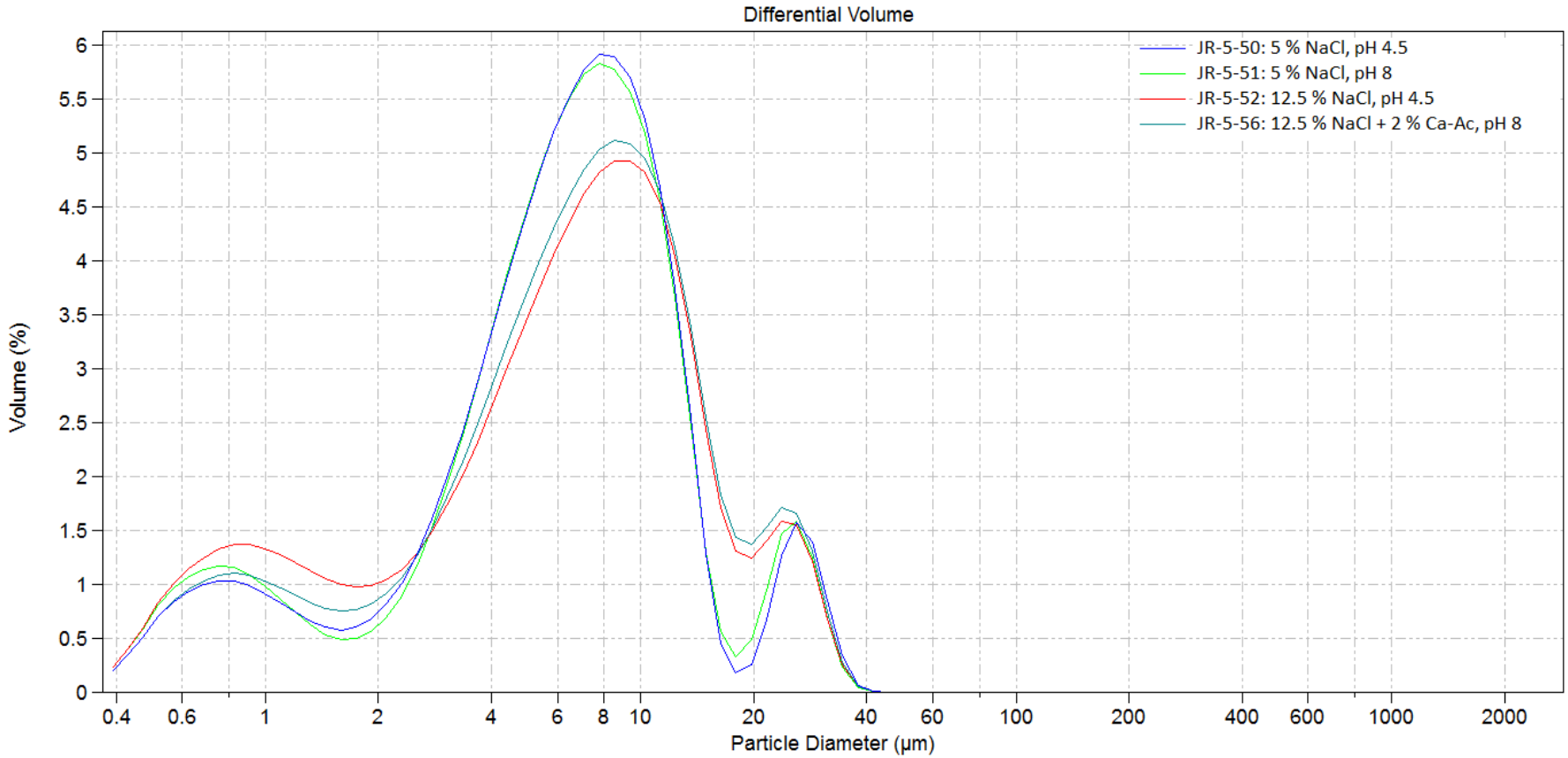


Figure 13. The particle size distribution curves of e*nzyme 3.3 samples did not show great differences between the samples with NaCl and the samples with also calcium acetate at different pH conditions.*

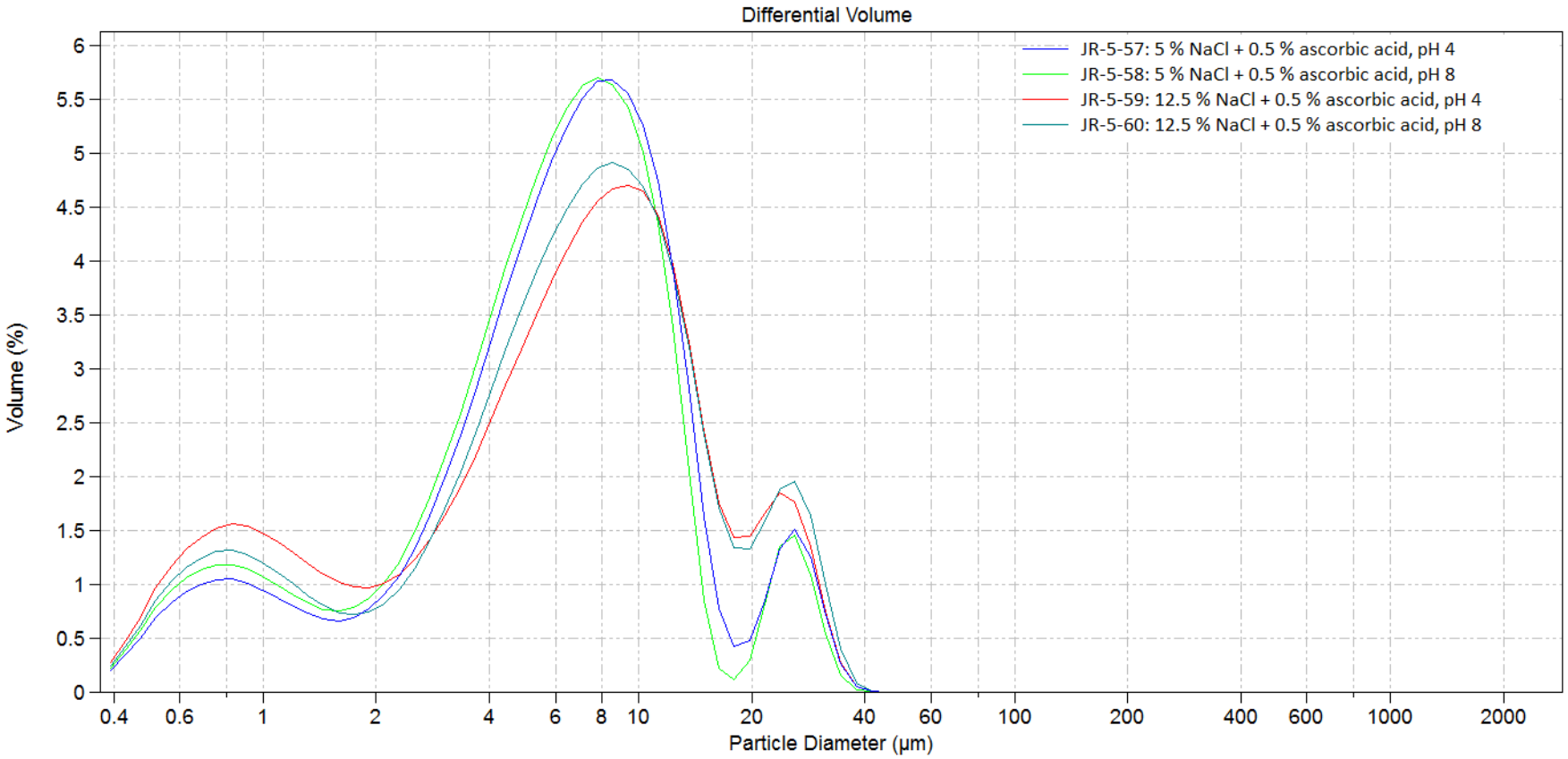


Figure 14. *Ascorbic acid was added to samples of enzyme 3.3 to be used together with NaCl and at different pH conditions. The particle size distribution curves looked similar but a slight decrease in the number of small particles was seen with samples JR-5-57 and JR-5-58.*

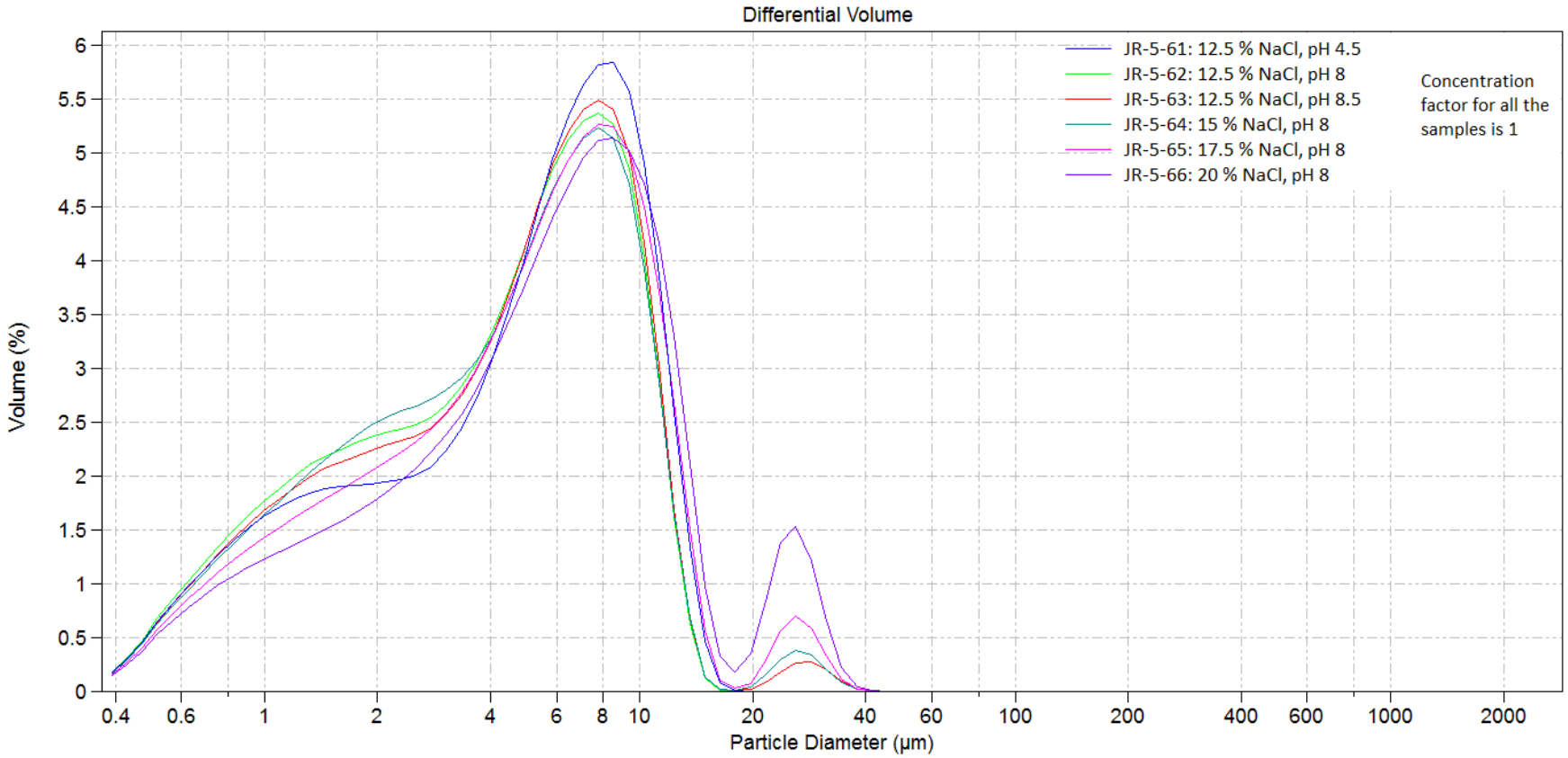


Figure 15. *Spray drying of enzyme 3.3 filtrate produced a great number of small particles and were not reduced by NaCl or varied pH conditions.*

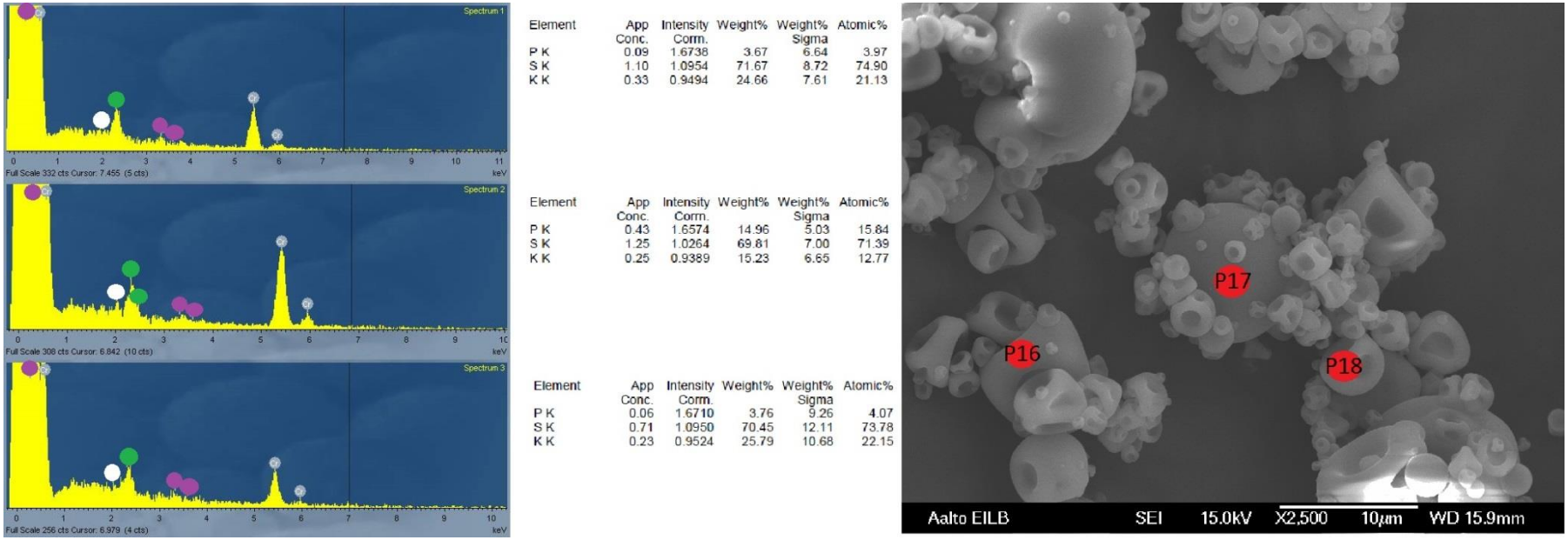
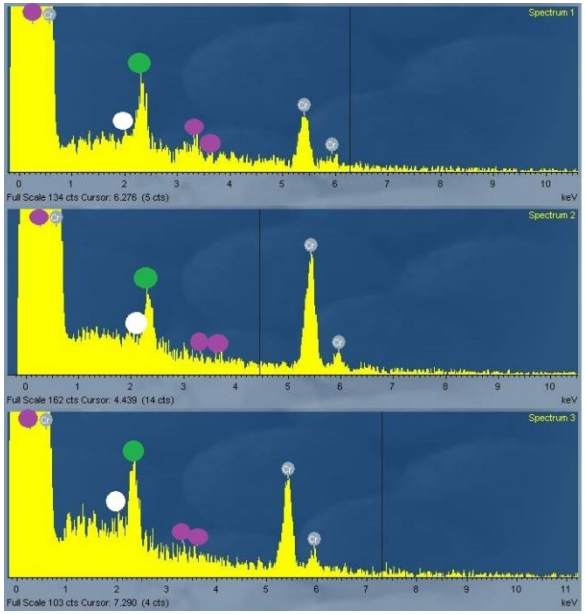


Figure 1. *The elemental spectrum of sample JR-2-2 (large cyclone, 140 µm nozzle cap) could only show the heavier elements than oxygen. Furthermore, the elements at low concentrations had relatively high error due to overlapping of the peaks. White: phosphorus; purple: potassium; green: sulphur; grey: chromium from sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
P K	0.19	1.6596	9.26	6.07	9.95
S K	0.89	1.0558	68.53	8.58	71.14
K K	0.26	0.9480	22.22	8.05	18.91

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
P K	0.23	1.6826	12.40	7.44	12.87
S K	0.96	1.0330	83.52	11.52	83.77
K K	0.04	0.9064	4.08	10.44	3.35

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
P K	0.24	1.6773	12.83	6.81	13.37
S K	0.94	1.0300	80.71	10.32	81.29
K K	0.07	0.9120	6.46	9.44	5.33

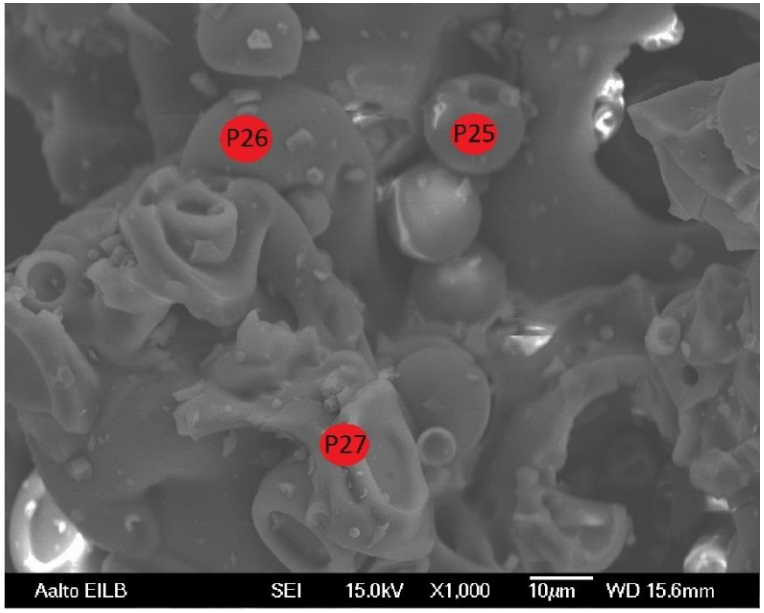


Figure 2. *The elemental spectrum for sample JR-2-5 (dried in production scale) did not give reliable results on phosphorus or potassium due to small peaks and strong overlapping. White: phosphorus; purple: potassium; green: sulphur; grey: chromium from sputtering.*

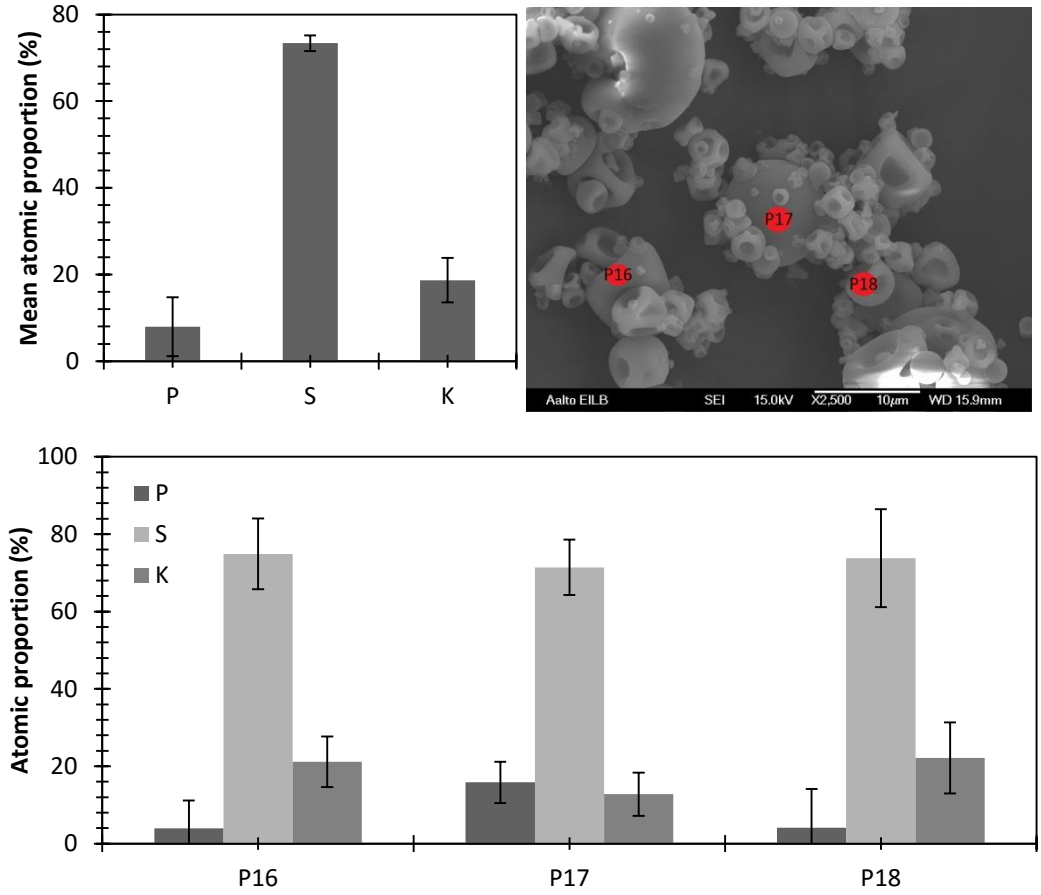


Figure 3. *The elemental analysis for sample JR-2-2 (large cyclone, 140 µm nozzle cap) showed differences in phosphorus and potassium concentrations but also high errors for them.*

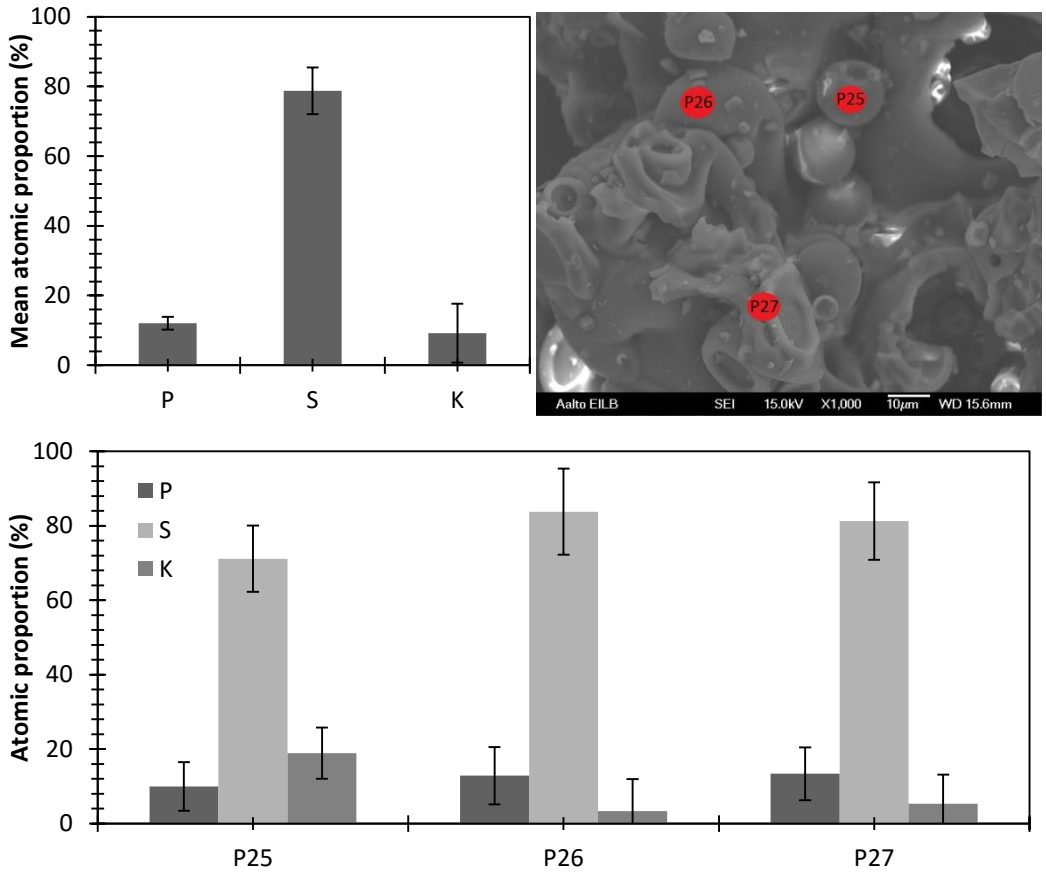


Figure 4. *The elemental analysis for sample JR-2-5 (dried in production scale) showed that the particle in the point P25 differs from the two other points. However, the errors in the measurements were too large to make any reliable conclusions.*

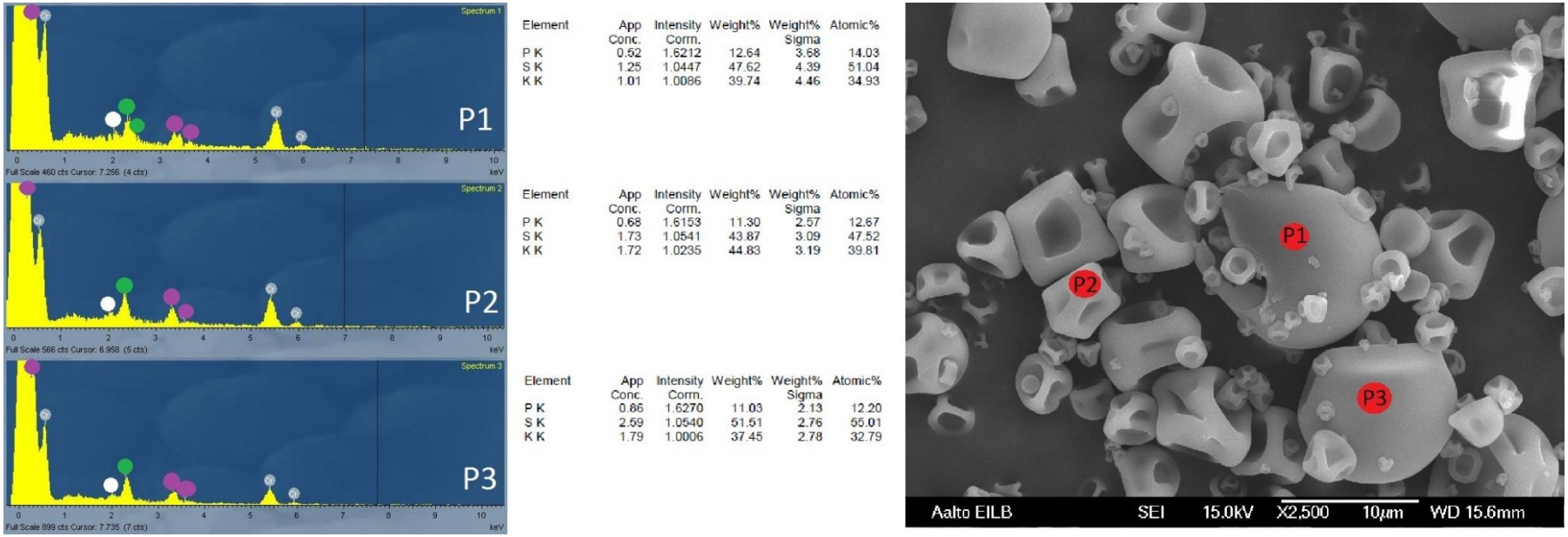


Figure 5. *Elemental analysis spectrum and data for JR-1-1 (non-concentrated enzyme solution, no additives) were similar for all the chosen particles. White: phosphorus; purple: potassium; green: sulphur; grey: chromium from sputtering.*

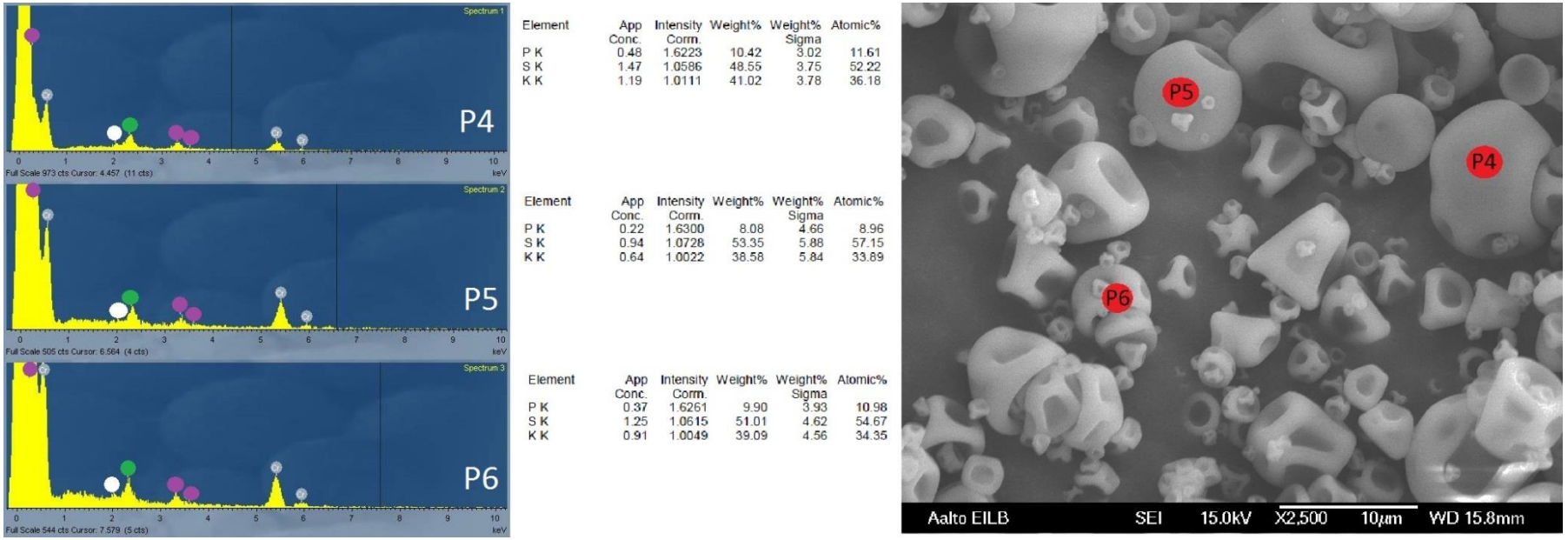


Figure 6. *The elemental analysis results for JR-1-2 suggested that different sized particles were similar. White: phosphorus; purple: potassium; green: sulphur; grey: chromium from sputtering.*

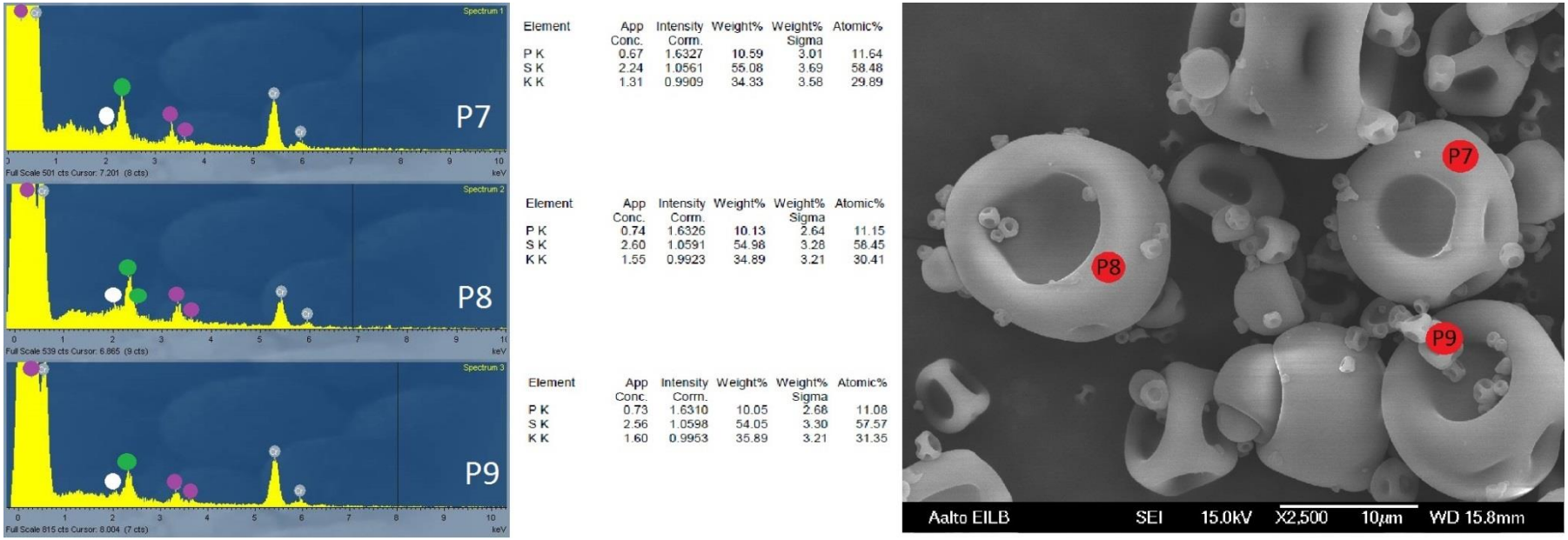
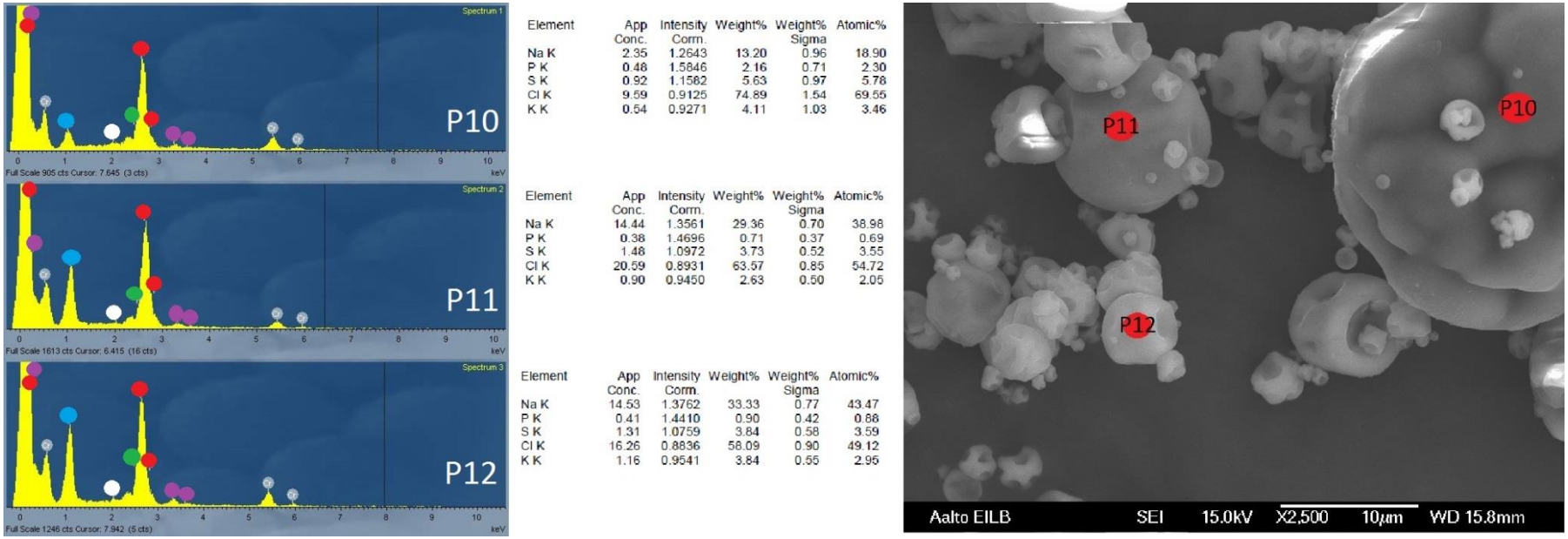


Figure 7. *The elemental analysis results for JR-1-3 suggested that different sized particles were similar .White: phosphorus; purple: potassium; green: sulphur; grey: chromium from sputtering.*

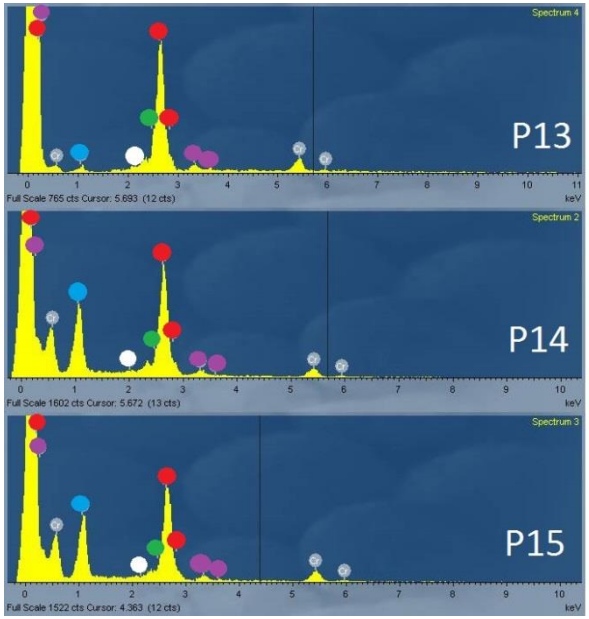


Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	2.35	1.2643	13.20	0.96	18.90
P K	0.48	1.5846	2.16	0.71	2.30
S K	0.92	1.1582	5.63	0.97	5.78
Cl K	9.59	0.9125	74.89	1.54	69.55
K K	0.54	0.9271	4.11	1.03	3.46

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	14.44	1.3561	29.36	0.70	38.98
P K	0.38	1.4696	0.71	0.37	0.69
S K	1.48	1.0972	3.73	0.52	3.55
Cl K	20.59	0.8931	63.57	0.85	54.72
K K	0.90	0.9450	2.63	0.50	2.05

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	14.53	1.3762	33.33	0.77	43.47
P K	0.41	1.4410	0.90	0.42	0.88
S K	1.31	1.0759	3.84	0.58	3.59
Cl K	16.26	0.8836	58.09	0.90	49.12
K K	1.16	0.9541	3.84	0.55	2.95

Figure 8. *The elemental analysis for sample JR-1-4 showed that there were differences in sodium and chlorine proportions in different particles. Smaller particle P12 had Na:Cl ratio closest to 1. Red: chlorine; blue: sodium; white: phosphorus; purple: potassium green: sulphur grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	0.55	1.2016	3.72	0.59	5.64
P K	-0.02	1.6672	-0.08	0.56	-0.09
S K	0.46	1.2443	3.04	0.92	3.31
Cl K	10.14	0.9565	86.35	1.51	84.93
K K	0.79	0.9213	6.97	1.10	6.21

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	14.47	1.3672	31.69	0.75	41.63
P K	0.48	1.4522	0.98	0.40	0.96
S K	1.39	1.0828	3.86	0.56	3.63
Cl K	17.73	0.8866	59.85	0.88	50.98
K K	1.15	0.9512	3.63	0.54	2.80

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	12.72	1.3655	32.09	0.79	42.09
P K	0.50	1.4501	1.19	0.42	1.16
S K	1.19	1.0801	3.80	0.58	3.58
Cl K	15.03	0.8864	58.56	0.92	49.81
K K	1.20	0.9537	4.35	0.57	3.35

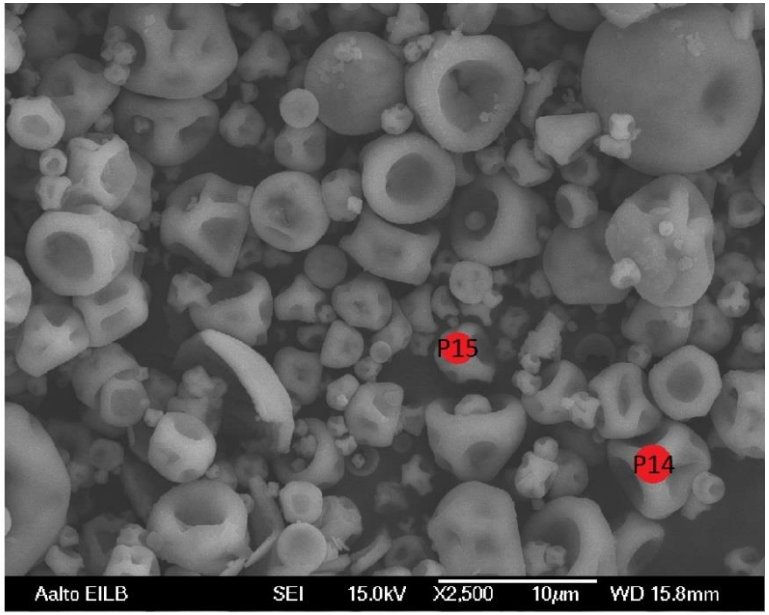
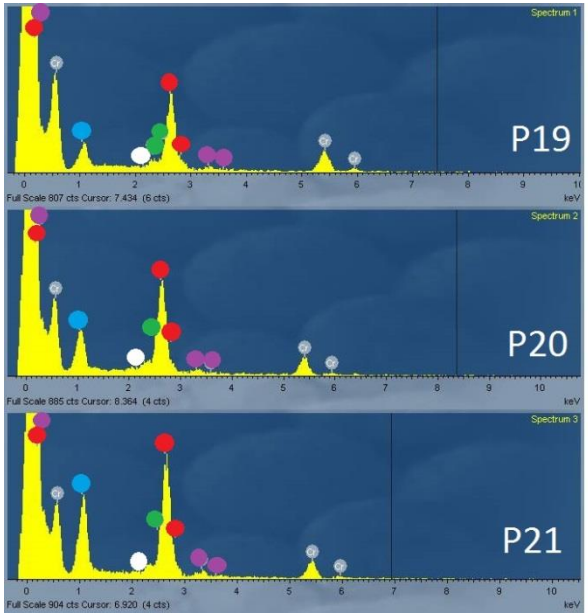


Figure 9. *The elemental analysis for sample JR-1-4 showed that particles P14 and P15 had similar elemental spectrums. P13 is missing from the picture Red: chlorine; blue: sodium; white: phosphorus; purple: potassium green: sulphur grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	2.54	1.2940	19.18	1.35	26.70
P K	0.14	1.5404	0.91	0.90	0.94
S K	0.72	1.1390	6.16	1.21	6.14
Cl K	6.43	0.9026	69.78	1.91	62.97
K K	0.38	0.9342	3.97	1.25	3.25

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	4.88	1.3278	24.51	1.14	33.23
P K	0.33	1.5037	1.47	0.70	1.48
S K	0.79	1.1124	4.72	0.97	4.59
Cl K	8.91	0.8964	66.41	1.50	58.40
K K	0.41	0.9389	2.89	0.96	2.30

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	9.46	1.3666	31.89	0.95	41.86
P K	0.30	1.4527	0.95	0.52	0.92
S K	0.87	1.0840	3.70	0.73	3.48
Cl K	11.52	0.8882	59.88	1.13	50.97
K K	0.74	0.9511	3.58	0.70	2.76

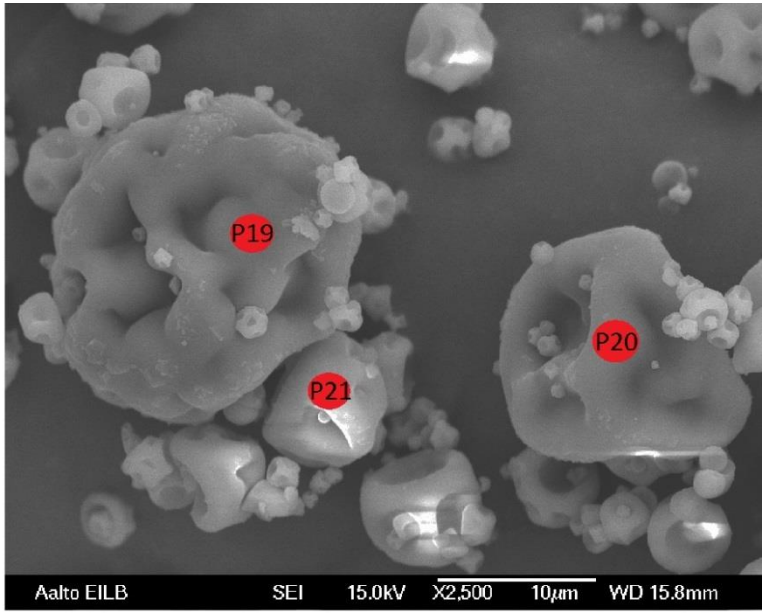
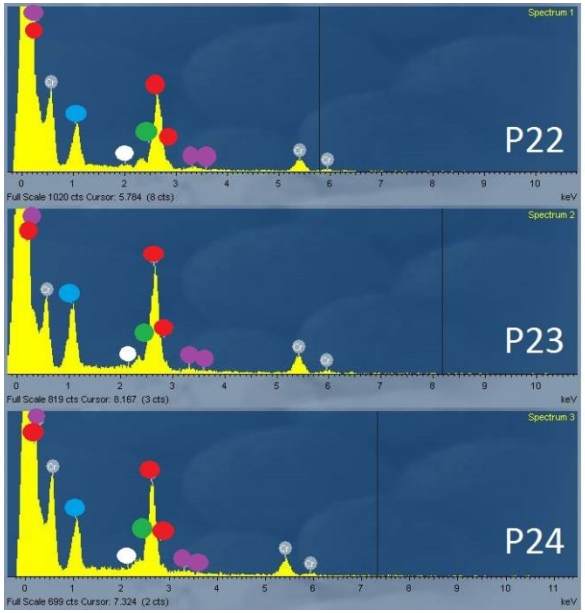


Figure 10. *The elemental analysis results for sample JR-1-5 suggested that larger particles had lower sodium and higher chlorine content. Red: chlorine; blue: sodium; white: phosphorus; purple: potassium green: sulphur grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	5.75	1.3496	27.65	1.15	36.84
P K	0.56	1.4795	2.44	0.69	2.41
S K	1.07	1.0861	6.40	0.95	6.12
Cl K	8.16	0.8775	60.52	1.42	52.29
K K	0.43	0.9439	3.00	0.89	2.35

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	7.11	1.3671	31.04	1.10	40.81
P K	0.31	1.4585	1.26	0.65	1.23
S K	0.94	1.0835	5.16	0.89	4.86
Cl K	8.82	0.8814	59.87	1.32	51.04
K K	0.42	0.9473	2.66	0.80	2.06

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	4.96	1.3639	29.63	1.33	39.18
P K	0.15	1.4707	0.82	0.83	0.81
S K	0.69	1.0954	5.12	1.12	4.85
Cl K	6.88	0.8861	63.43	1.67	54.39
K K	0.11	0.9404	0.99	1.07	0.77

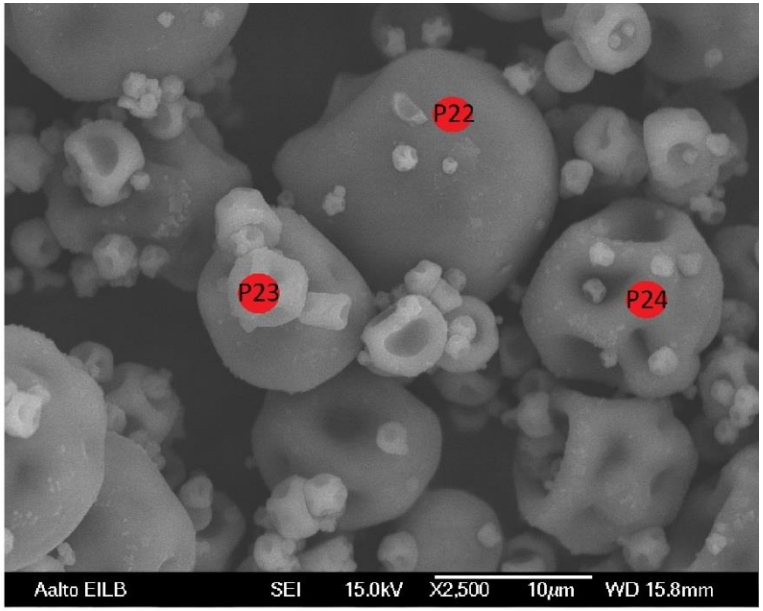


Figure 11. *The elemental analysis results from JR-1-6 suggested that smaller particles had higher sodium and lower chlorine content compared to larger particles. Red: chlorine; blue: sodium; white: phosphorus; purple: potassium green: sulphur grey: chromium from the sputtering.*

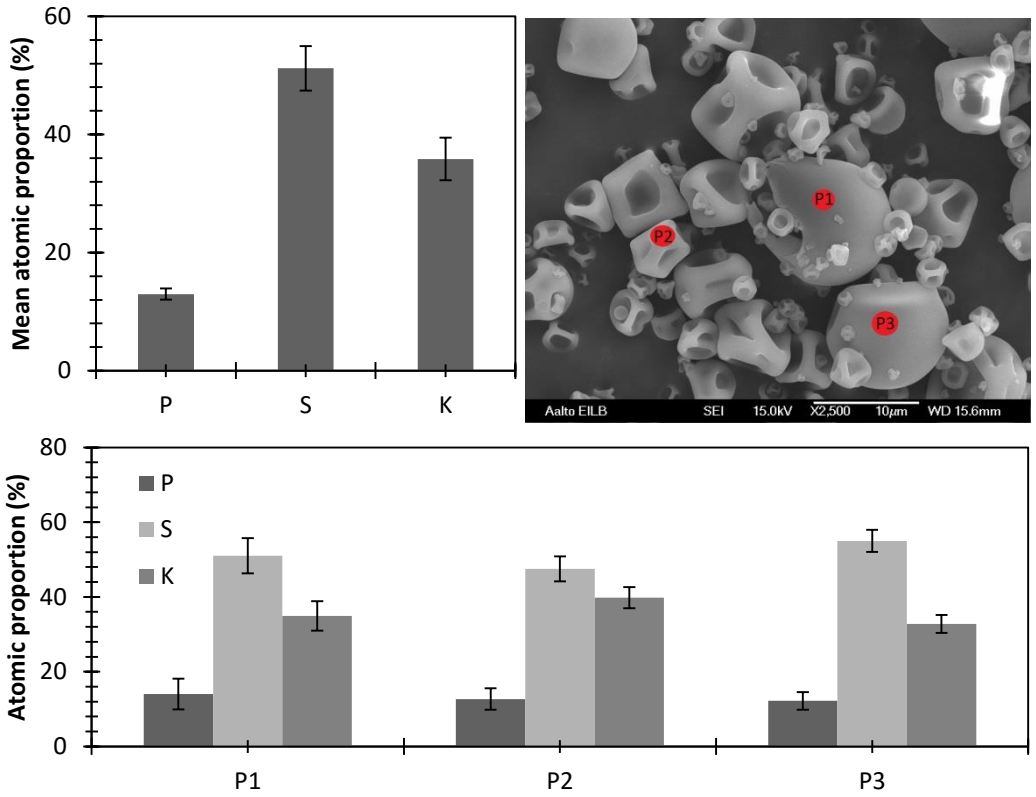


Figure 12. *Elemental analysis results did not show significant differences between particles P1-P3 in sample JR-1-1.*

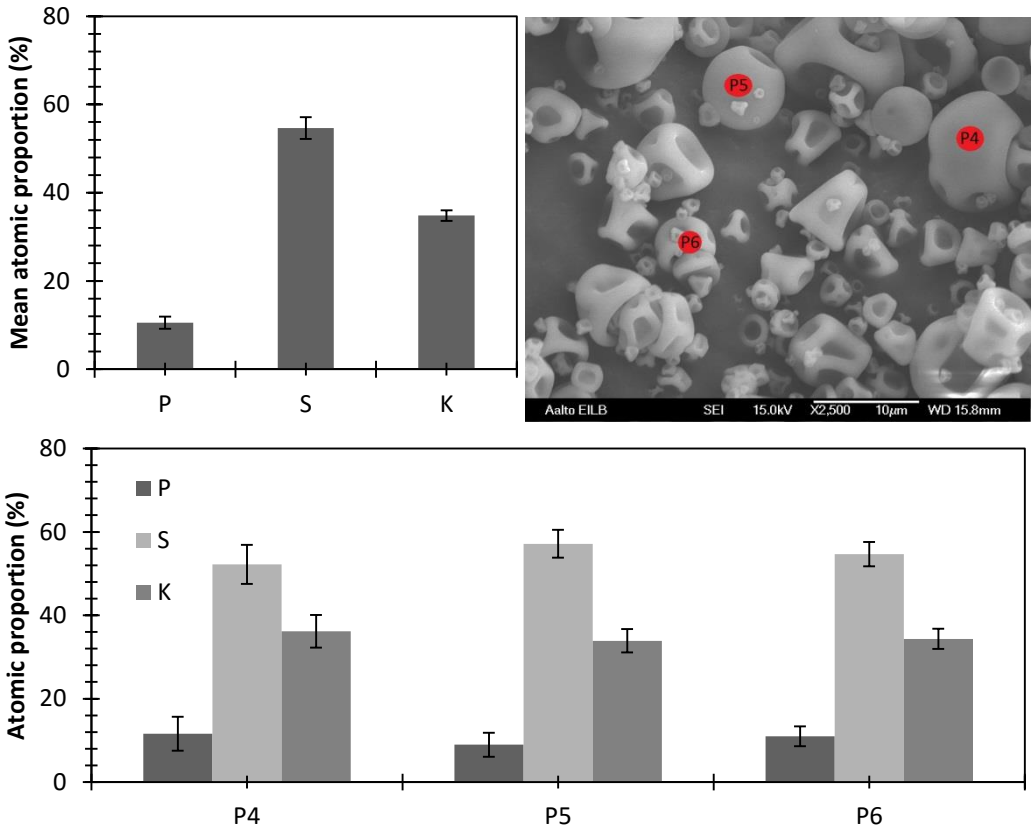


Figure 13. *Different sized particles seemed to have similar atomic proportions for sample JR-1-2.*

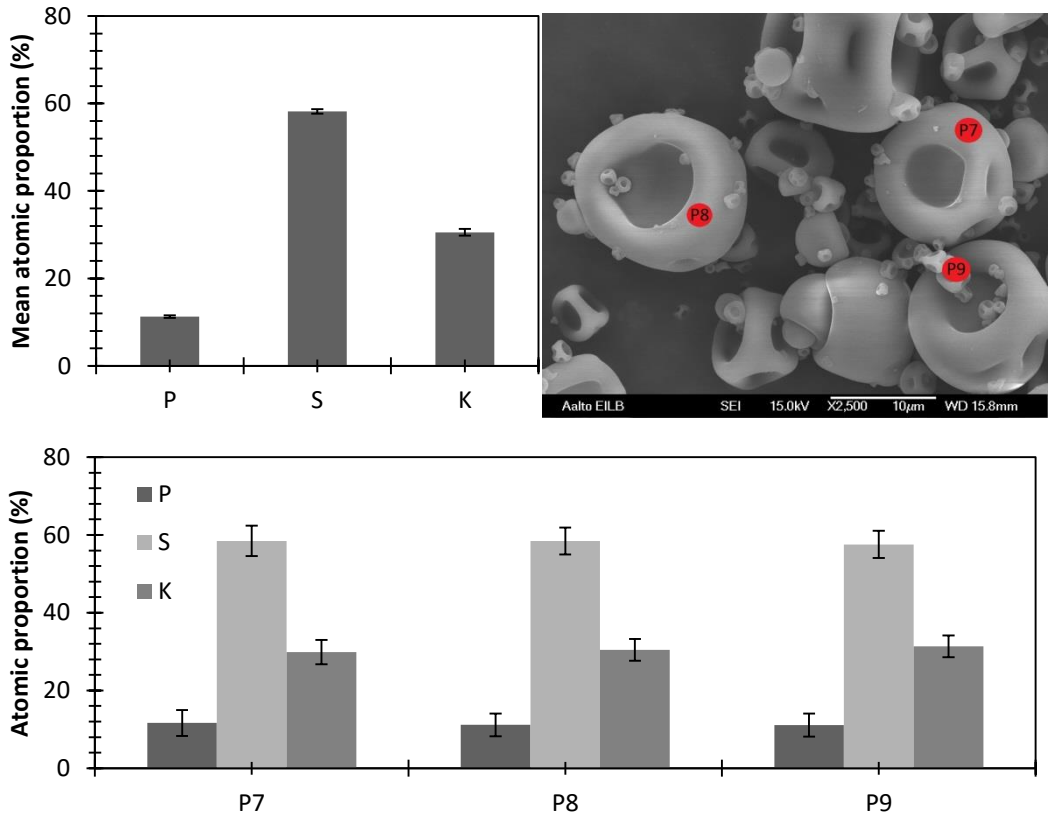


Figure 14. *Particles in sample JR-1-3 were very similar according to elemental analysis.*

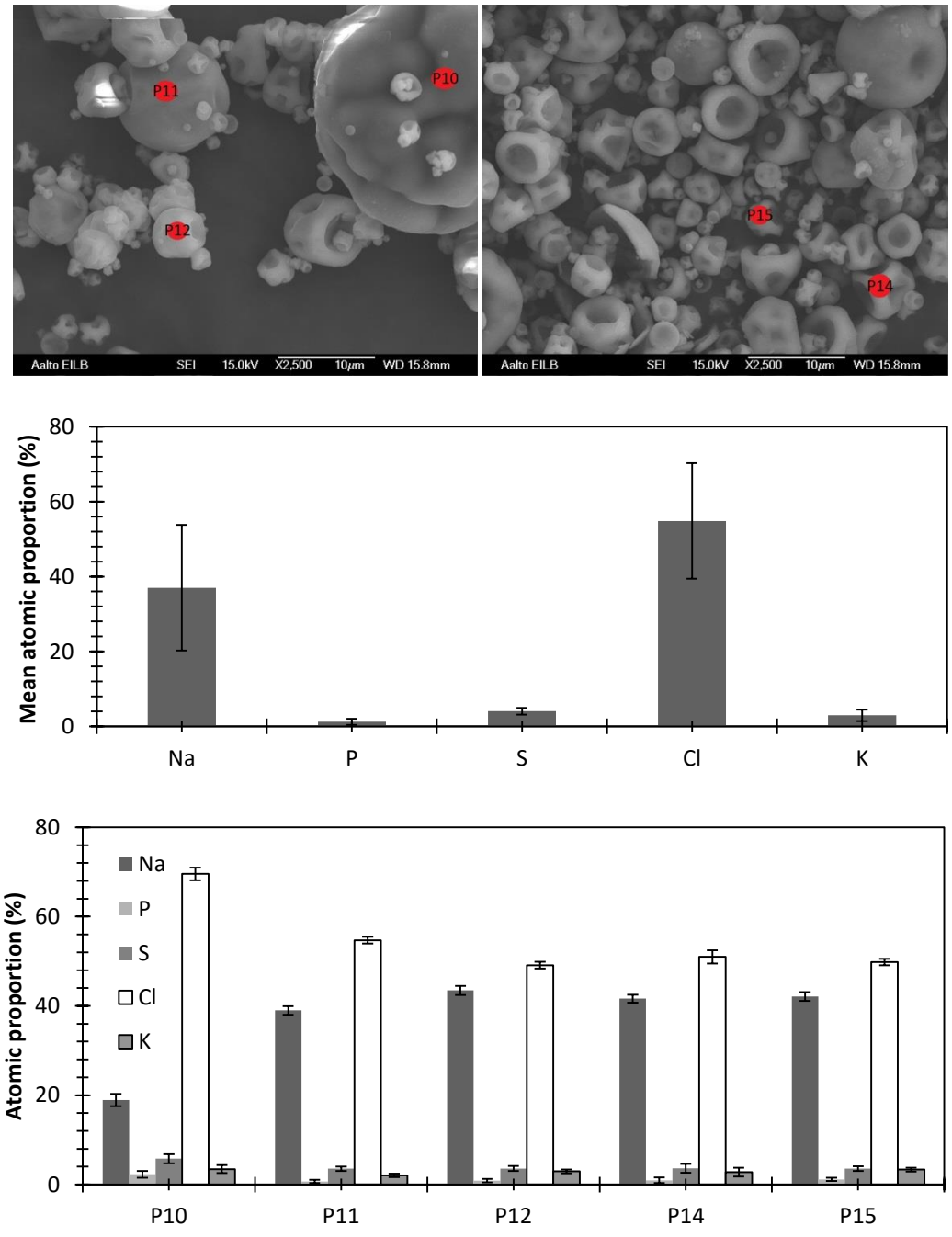


Figure 15. *In sample JR-1-4 sodium and chlorine proportions varied significantly between different particles.*

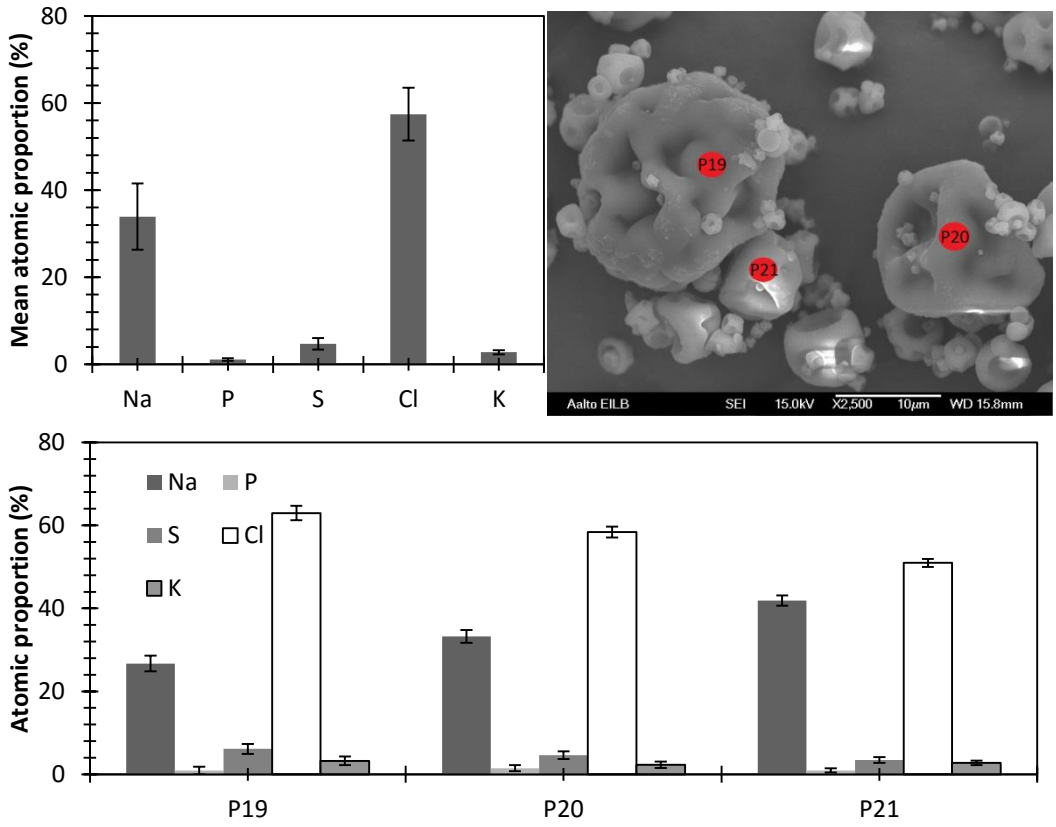


Figure 16. *The elemental analysis of sample JR-1-5 suggested that different sized particles had different atomic content. It seemed like the ratio between sodium and chlorine approached 1:1 when particle size decreased.*

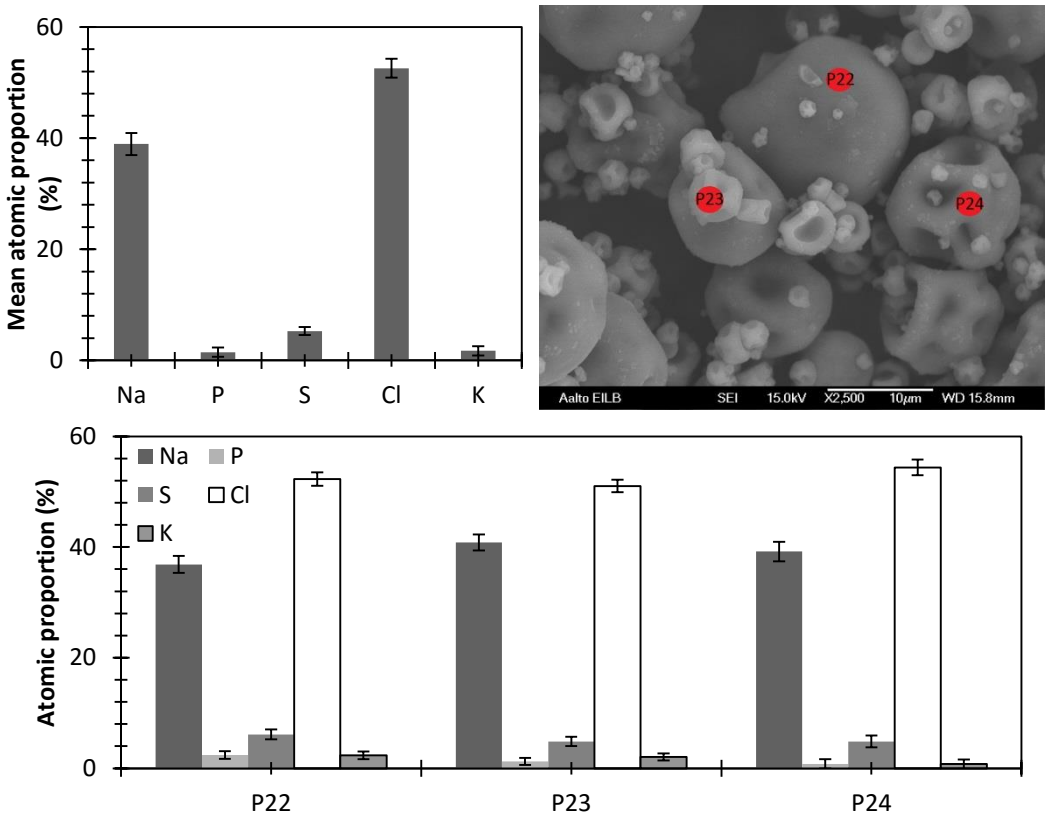
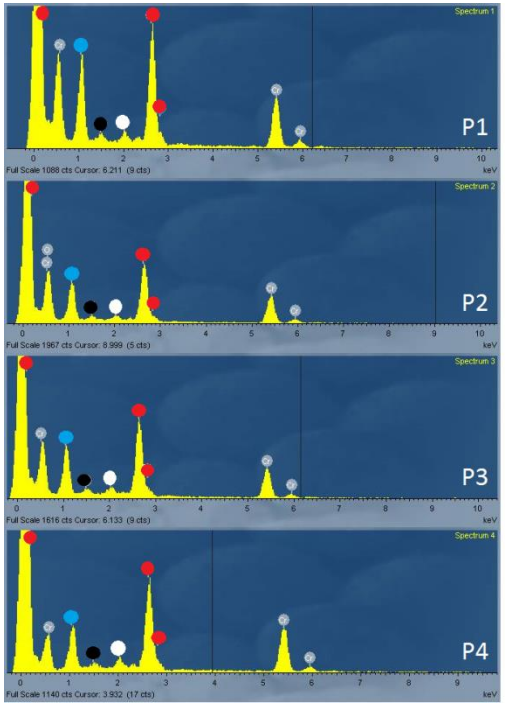


Figure 17. *Sample JR-1-6 showed differences in particle elemental content where larger particles P22 and P24 showed lower proportion of sodium and lower chlorine compared to smaller particle P23. Also, particles P22 and P24 had different morphology but elemental analysis results looked similar.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	13.05	1.4100	33.69	0.80	43.42
Al K	0.63	0.8829	2.61	0.48	2.87
P K	1.49	1.4198	3.82	0.51	3.66
Cl K	14.51	0.8831	59.87	0.88	50.05
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	10.95	1.4005	32.09	0.85	41.65
Al K	0.63	0.8931	2.91	0.52	3.22
P K	1.24	1.4277	3.56	0.55	3.43
Cl K	13.25	0.8866	61.44	0.95	51.71
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	11.28	1.3966	30.95	0.81	40.31
Al K	0.78	0.9010	3.33	0.49	3.69
P K	1.48	1.4309	3.98	0.54	3.85
Cl K	14.25	0.8861	61.74	0.91	52.15
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	7.04	1.3679	26.41	0.89	35.15
Al K	0.42	0.9316	2.31	0.54	2.62
P K	1.63	1.4701	5.69	0.63	5.62
Cl K	11.39	0.8917	65.60	1.03	56.62
Totals			100.00		

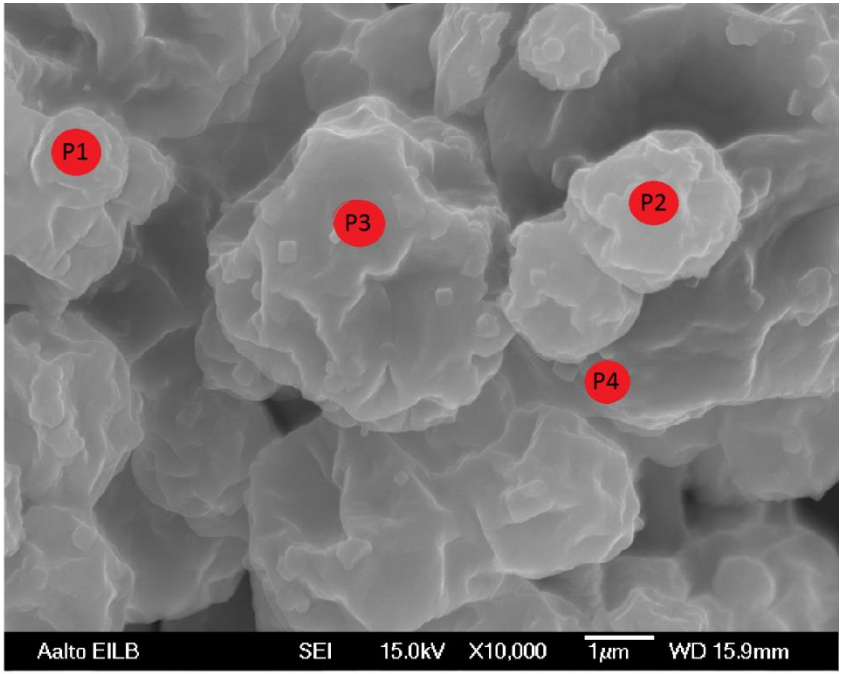
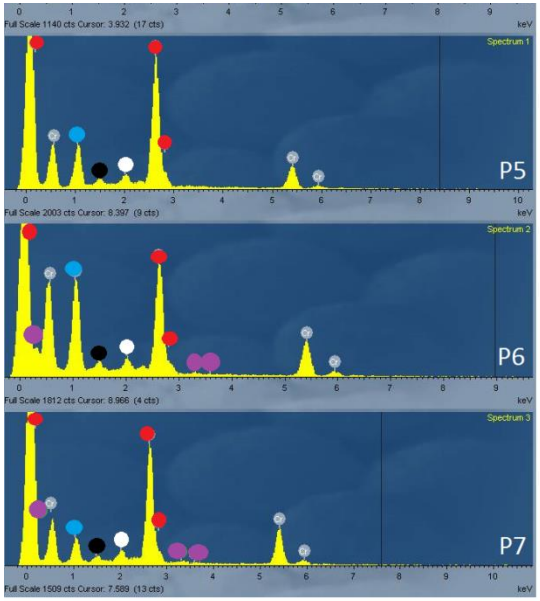


Figure 18. *Elemental analysis spectrums and results of sample JR-5-2 (5 % NaCl, pH 5) with 10 000x magnification were taken from several points to get more reliable results. However, P4 gives differing results. Red: chlorine; blue: sodium; black: aluminum; white: phosphorus; grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	11.34	1.3302	20.31	0.54	27.87
Al K	0.94	0.9732	2.31	0.31	2.70
P K	2.86	1.5125	4.50	0.38	4.59
Cl K	27.78	0.9084	72.88	0.65	64.85
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	22.67	1.4242	35.64	0.64	45.44
Al K	1.28	0.8703	3.28	0.37	3.57
P K	3.04	1.3988	4.87	0.41	4.61
Cl K	21.43	0.8729	55.01	0.71	45.49
K K	0.51	0.9554	1.19	0.38	0.90
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	4.90	1.2901	14.02	0.64	19.83
Al K	0.72	1.0190	2.60	0.37	3.13
P K	2.17	1.5546	5.14	0.50	5.40
Cl K	19.03	0.9194	76.41	0.92	70.11
K K	0.46	0.9265	1.83	0.58	1.53
Totals			100.00		

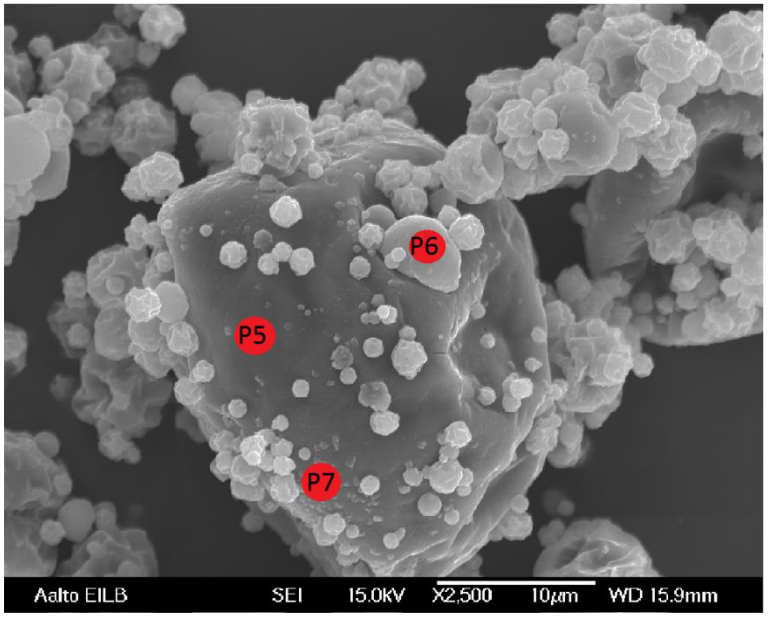
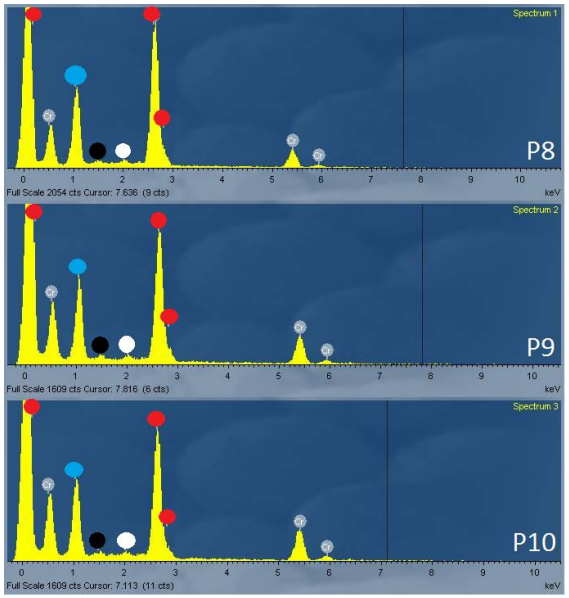


Figure 19. *Clearly differing particle from the sample JR-5-2 (5 % of NaCl, pH 5) was found and analyzed. Red: chlorine; blue: sodium; black: aluminum; white: phosphorus; purple: potassium; grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	20.66	1.3737	30.36	0.54	40.04
Al K	0.39	0.9019	0.87	0.26	0.97
P K	1.02	1.4613	1.41	0.27	1.38
Cl K	30.16	0.9053	67.36	0.59	57.61
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	16.20	1.3871	31.96	0.66	41.76
Al K	0.46	0.8921	1.40	0.34	1.56
P K	0.91	1.4448	1.73	0.35	1.68
Cl K	21.29	0.8989	64.91	0.72	55.00
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	16.20	1.3860	31.95	0.66	41.78
Al K	0.44	0.8920	1.36	0.35	1.51
P K	0.75	1.4455	1.42	0.36	1.38
Cl K	21.46	0.9003	65.27	0.72	55.33
Totals			100.00		

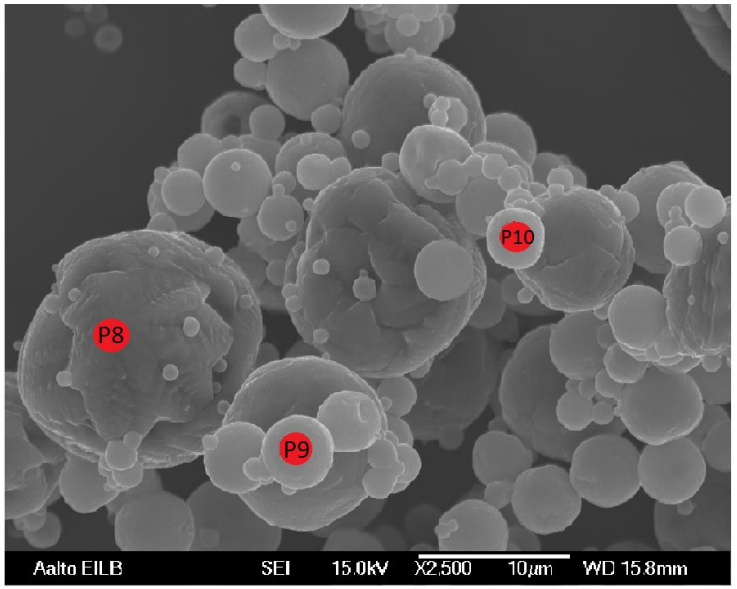
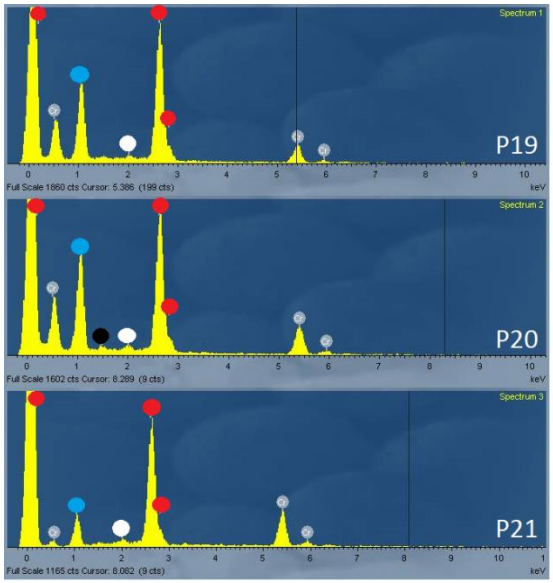


Figure 20. *The elemental analysis spectrums and results of the sample JR-5-5 (12.5 % of NaCl, pH 8) were slightly different to different type of particles (P8 = large, not smooth; P9 and P10 = smaller and smooth spheres). Red: chlorine; blue: sodium; black: aluminum; white: phosphorus; grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	18.83	1.3787	31.88	0.59	41.85
P K	0.85	1.4595	1.37	0.31	1.33
Cl K	25.82	0.9048	66.75	0.62	56.82
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	20.14	1.4039	35.02	0.63	45.19
Al K	0.33	0.8725	0.93	0.31	1.03
P K	0.97	1.4288	1.66	0.32	1.59
Cl K	22.81	0.8941	62.38	0.67	52.19
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	4.71	1.3012	18.30	0.71	25.60
P K	0.61	1.5583	1.99	0.47	2.07
Cl K	14.67	0.9316	79.71	0.80	72.33
Totals			100.00		

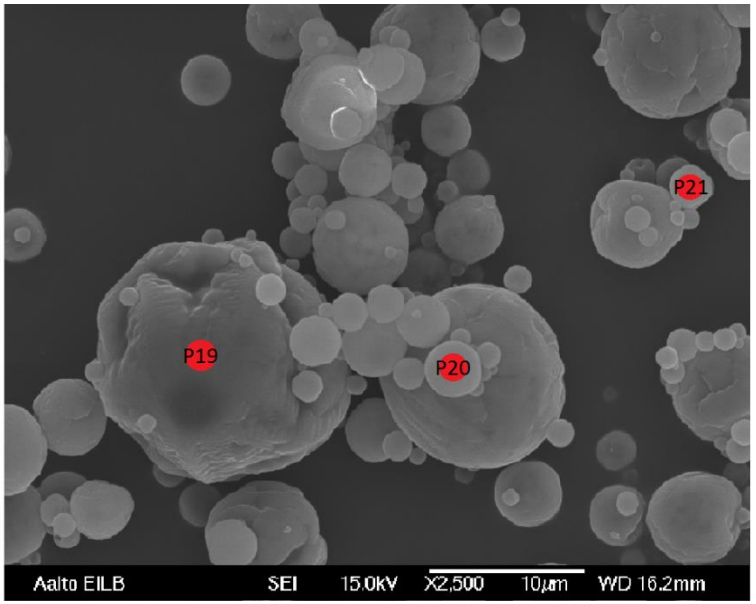
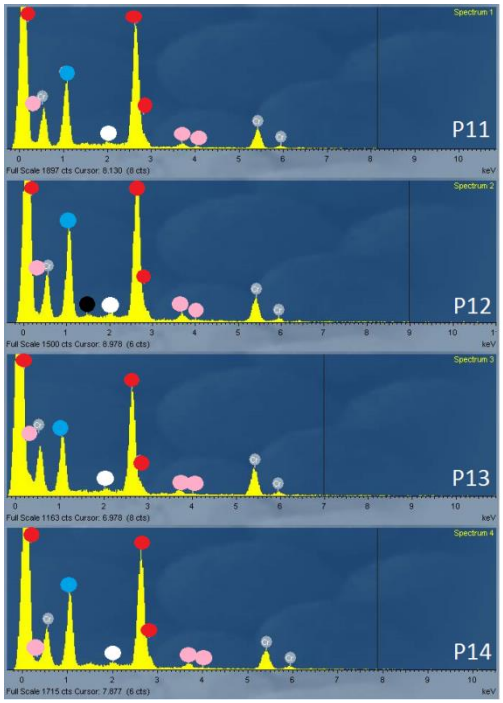


Figure 21. *The elemental analysis spectrums and results of the sample JR-5-17 (15 % of NaCl, pH 8) showed the differences between different particles. Red: chlorine; blue: sodium; black: aluminum; white: phosphorus; purple: potassium; grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	17.69	1.3429	29.63	0.58	39.45
P K	0.73	1.4678	1.13	0.29	1.11
Cl K	26.64	0.9097	65.95	0.65	56.93
Ca K	1.37	0.9376	3.29	0.39	2.51
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	19.35	1.3547	31.57	0.59	41.56
Al K	0.34	0.8866	0.84	0.28	0.94
P K	1.04	1.4441	1.59	0.30	1.56
Cl K	25.21	0.9008	61.95	0.67	52.88
Ca K	1.72	0.9418	4.05	0.41	3.05
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	9.35	1.3384	29.41	0.83	39.18
P K	0.67	1.4667	1.91	0.44	1.89
Cl K	13.89	0.9066	64.57	0.94	55.79
Ca K	0.92	0.9393	4.11	0.60	3.14
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	18.04	1.3633	32.88	0.63	43.08
P K	0.98	1.4449	1.68	0.31	1.64
Cl K	22.46	0.9004	62.08	0.69	52.76
Ca K	1.27	0.9411	3.36	0.43	2.52
Totals			100.00		

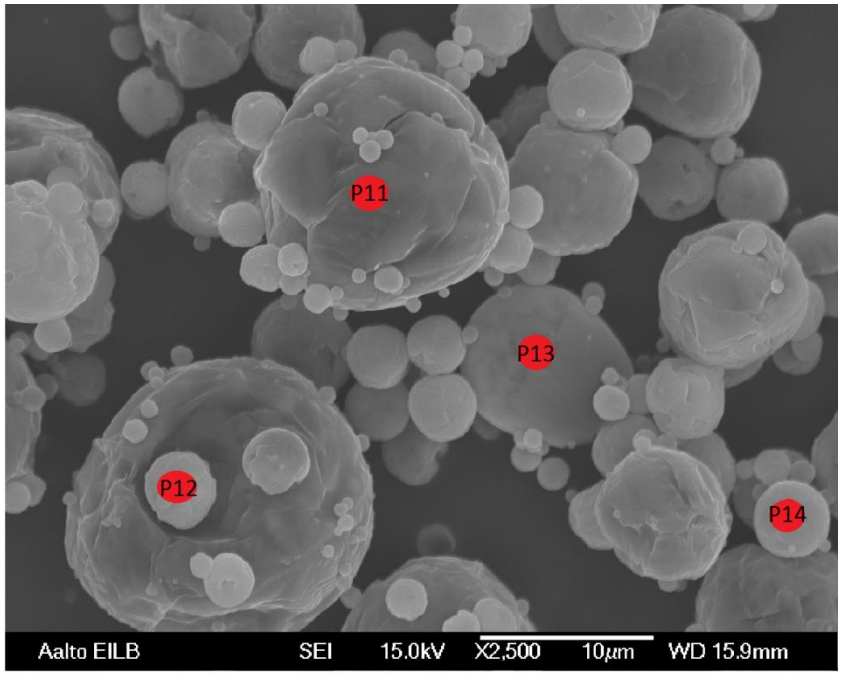
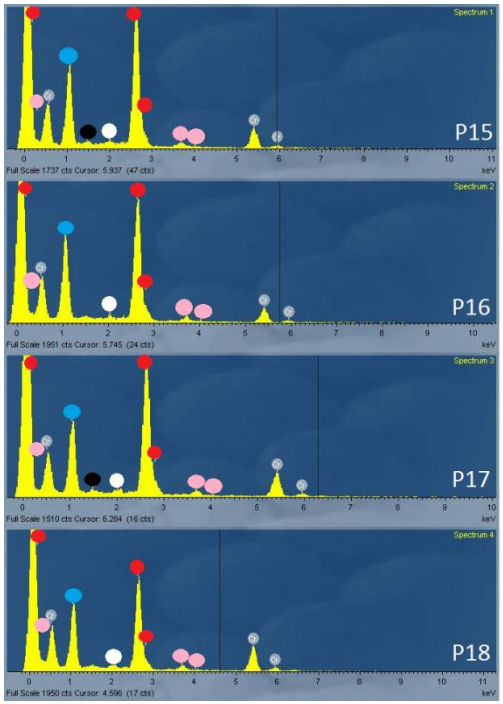


Figure 22. *The elemental analysis spectrums and results of the sample JR-5-9 (12.5 % of NaCl + 2 % Ca-acetate, pH 4.5) suggested that particles at points P11 and P13, and P12 and P14 were similar. Red: chlorine; blue: sodium; black: aluminum; white: phosphorus; rose: calcium; grey: chromium from the sputtering.*



Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	20.00	1.3605	31.36	0.58	41.29
Al K	0.35	0.8901	0.85	0.27	0.95
P K	0.84	1.4485	1.24	0.29	1.21
Cl K	26.95	0.9031	63.73	0.66	54.42
Ca K	1.24	0.9390	2.83	0.39	2.13
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	23.07	1.3703	34.10	0.57	44.46
P K	0.87	1.4357	1.23	0.28	1.19
Cl K	27.17	0.8991	61.22	0.62	51.77
Ca K	1.61	0.9425	3.46	0.37	2.58
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	15.88	1.3476	29.67	0.62	39.38
Al K	0.35	0.8999	0.99	0.29	1.12
P K	0.87	1.4570	1.50	0.31	1.48
Cl K	23.11	0.9050	64.37	0.72	55.39
Ca K	1.29	0.9388	3.46	0.43	2.64
Totals			100.00		

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
Na K	18.26	1.3658	34.40	0.65	44.82
P K	0.90	1.4310	1.62	0.34	1.57
Cl K	20.70	0.8965	59.40	0.72	50.19
Ca K	1.68	0.9451	4.58	0.46	3.42
Totals			100.00		

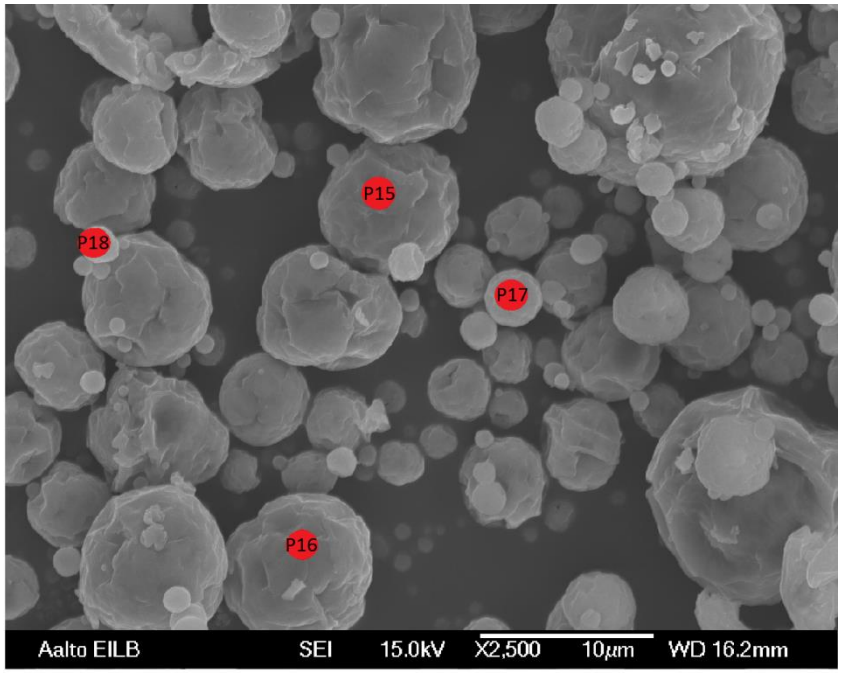


Figure 23. *The elemental analysis spectrums and results of the sample JR-5-14 (12.5 % of NaCl + 2 % Ca-acetate, pH 8) suggested that particles at points P15 and P17, and P16 and P18 were similar, but the picture showed different. Red: chlorine; blue: sodium; black: aluminum; white: phosphorus; rose: calcium; grey: chromium from the sputtering.*

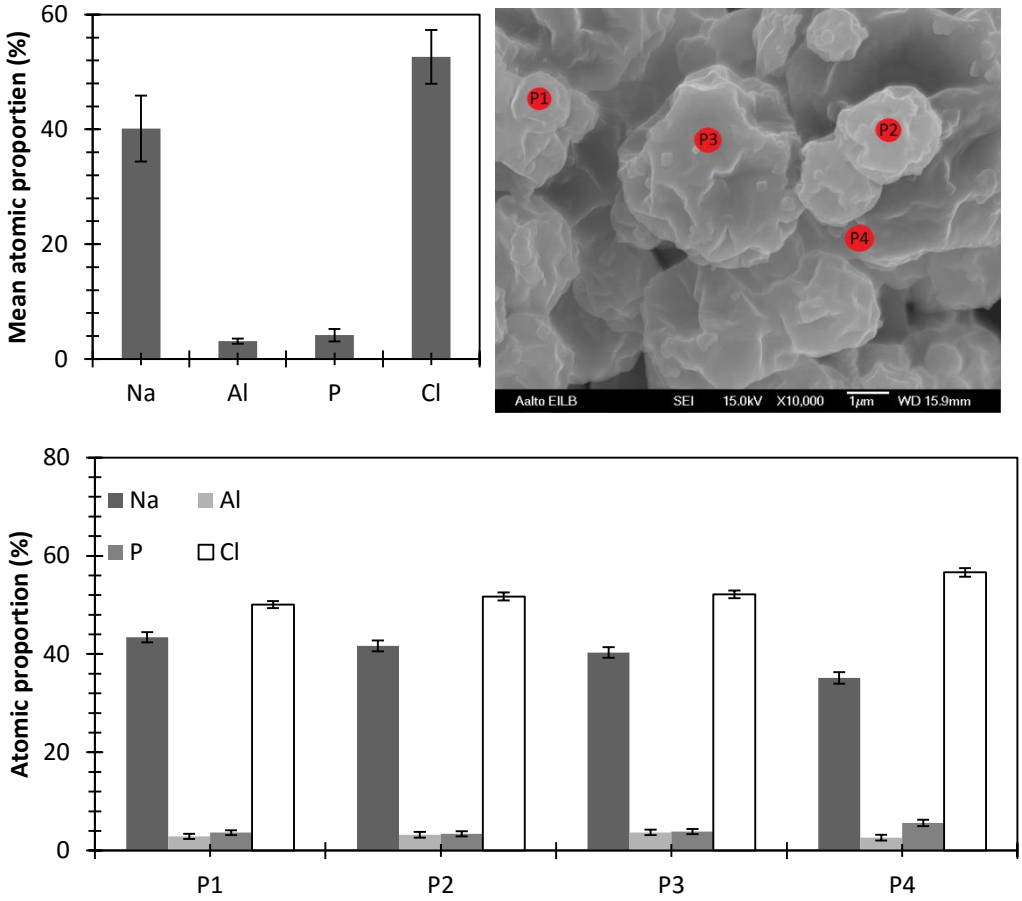


Figure 274. *Atomic proportion (%) of four analysis points of sample JR-5-2 were measured as well as mean atomic proportion of sodium, aluminum, phosphorus and chloride.*

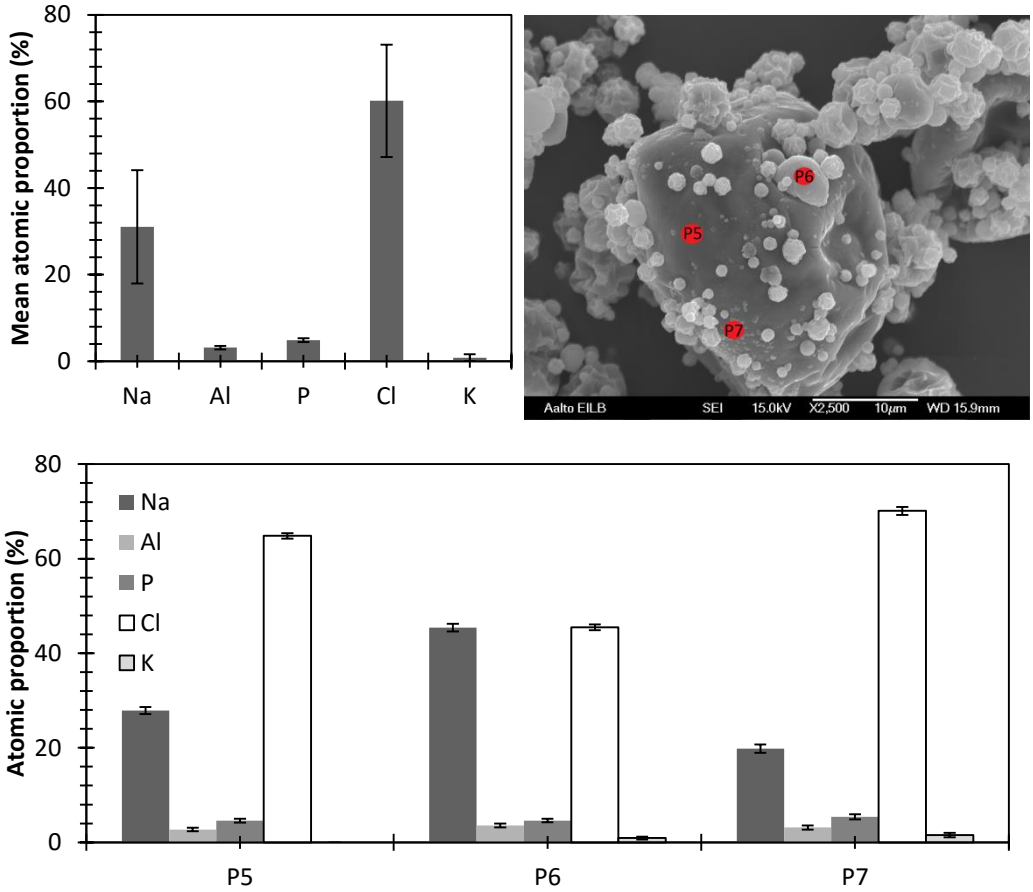


Figure 285. *Atomic proportion (%) of three analysis points of sample JR-5-2 was measured of differing particle. Also mean atomic proportion of sodium, aluminum, phosphorus, chloride and potassium was analyzed.*

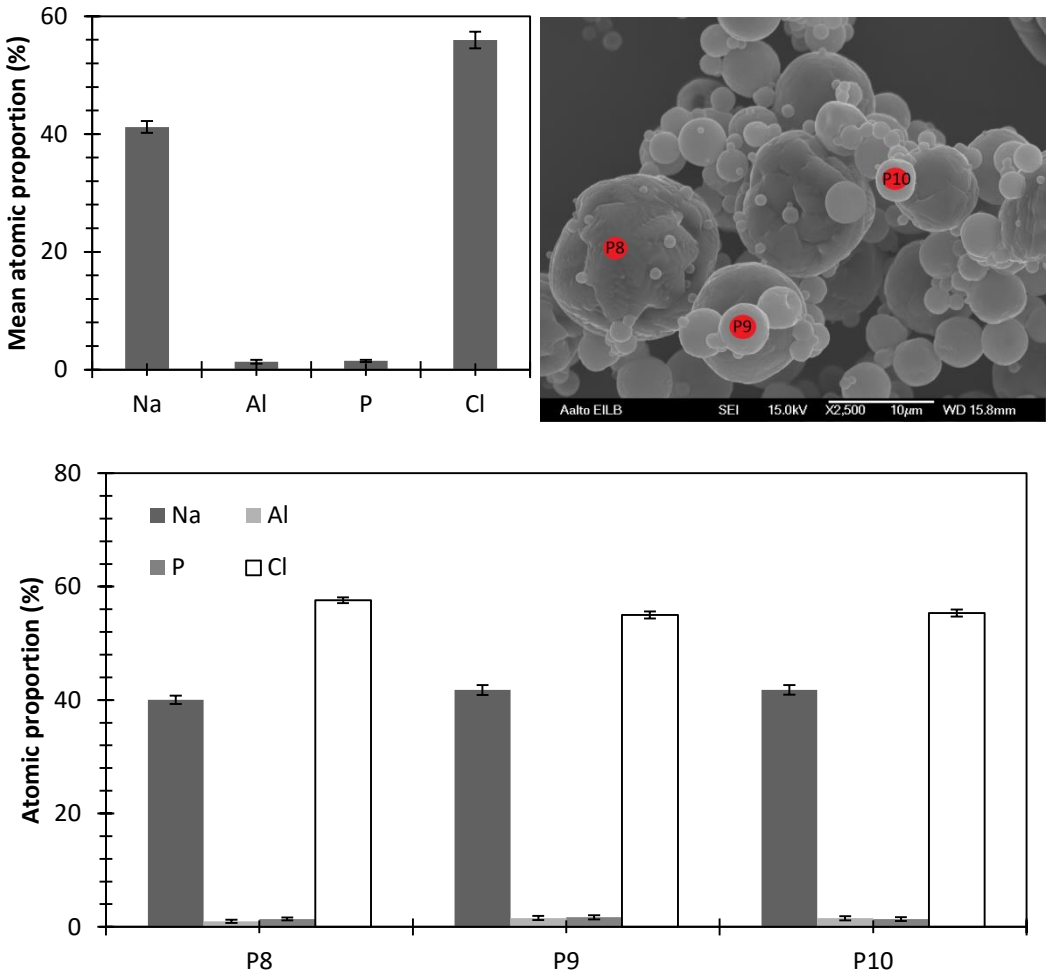


Figure 26. *The results from the elemental analysis of sample JR-5-5 did not show significant differences between different particles.*

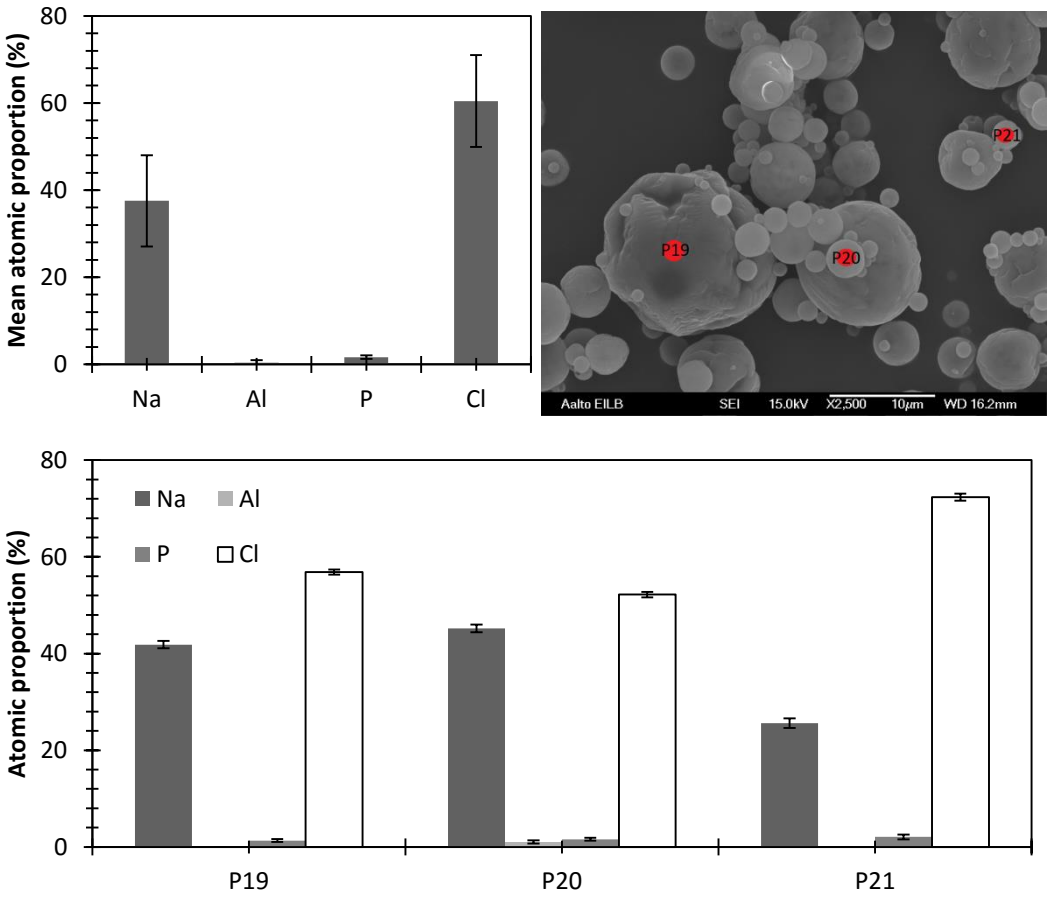


Figure 27. *Atomic proportion (%) of three analysis points of sample JR-5-17 was measured. Also mean atomic proportion of sodium, aluminum, phosphorus and chloride was analyzed.*

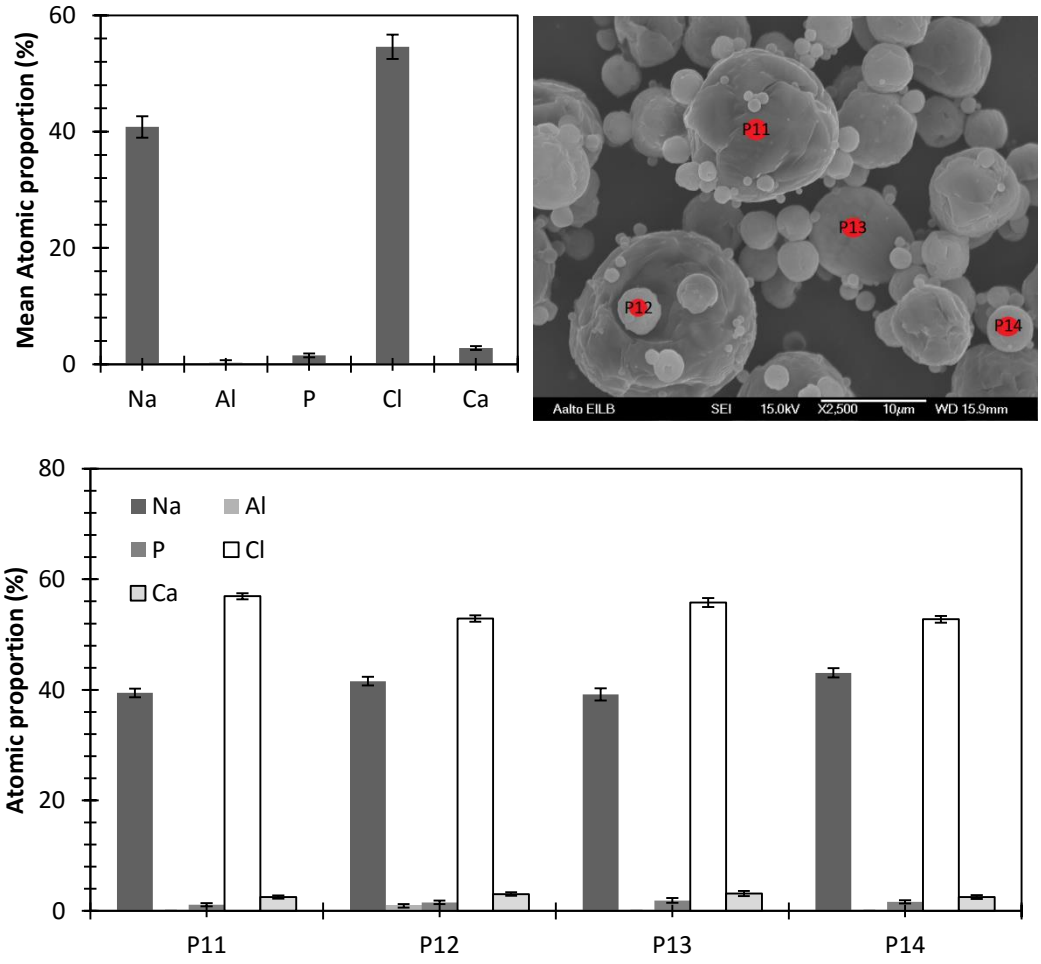


Figure 28. *The elemental analysis for the sample JR-5-9 showed aluminum in P12 but the error is relatively large.*

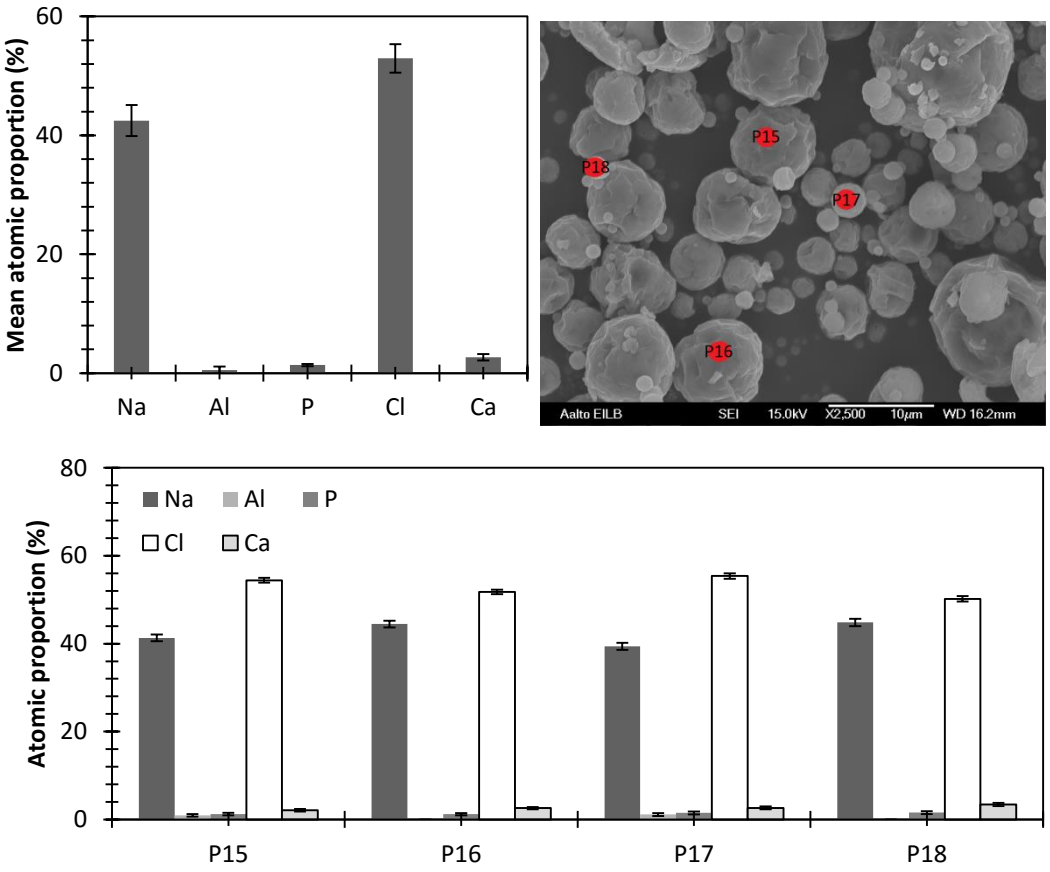


Figure 29. *The results of the sample JR-5-14 suggested that there were only small differences in the elemental proportions in different particles. Also points P15 and P17 had small amounts of aluminum.*

Table 17. *Results of enzyme 1B activities had problems with the analysis as the activity yields either are above 100 % or have no consistency. The patterns could be analyzed for samples with NaCl, and it seemed like the samples with NaCl had higher activity yields than without NaCl. Methyl cellulose (MC) did not show the same effect.*

	NaCl conc.		**Na_2SO_4 conc.**			
Enzyme solution conc.	**0 %**	**6.8 %**	**6.80 %**	**6.0 %**	**5 %**	**4 %**
Filtrate	90.3 %	103.5 %	64.8 %			
Mix	84.9 %	100.4 %	103.3 %			
Concentrate	89.1 %	106.5 %	97.4 %	124.6 %	241.1 %	100.4 %
	MC 1,5 %	86.6 %				